BHATT, ISHAN SUNILKUMAR, Ph.D. A Polymorphism in Human Estrogen-Related Receptor Beta (ESRRβ) is Associated with Physiological Measures of Noise-Induced Hearing Loss. (2013)
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Noise-induced hearing loss (NIHL) is a common form of hearing loss and a growing health concern despite national standards for hearing protection and public health awareness campaigns. An NIHL gene association study with college-aged musicians has associated a non-synonymous single nucleotide polymorphism (rs61742642; C→T, P386S) in the ligand-binding domain of human estrogen-related receptor beta (ESRRβ) with increased susceptibility to bilateral 4 to 6 kHz hearing loss. ESRRβ protein is expressed in major cochlear structures except hair cells and tectorial membrane. ESRRβ controls epithelial cell fate and endolymph production in the stria vascularis by regulating genes responsible for potassium ion transportation. Mutation in ESRRβ gene is associated with autosomal-recessive nonsyndromic profound hearing loss. The purpose of the study was to examine the effects of the ESRRβ polymorphism on temporary NIHL in young individuals.

Methods: 19 individuals with rs61742642 CT genotype and 40 individuals with rs61742642 CC genotype were recruited for the study. Temporary NIHL was induced by 10 minutes exposure to 90 dB SL 2 kHz audiometric narrow-band noise and cochlear physiology was evaluated by a battery of clinical tests consisting audiometry, distortion product of otoacoustic emission (DPOAE) and transient evoked otoacoustic emissions (TEOAE). Input-output function of distortion product of OAE (DPOAE) was collected before and after the noise exposure using L1 = (0.40) L2 +39 at 2, 3 and 4 kHz. TEOAEs

were collected using ILO quickscreen protocol with 84 dB peSLP with and without 50 dB SL contralateral broadband noise. Audiometric temporary threshold shift (ATTS), DPOAE temporary level shift (DPTLS), TEOAE temporary level shift (TETLS), TEOAE temporary level shift (TETLS) and TEOAE temporary suppression shift (TETSS) were evaluated to explore physiological basis of NIHL susceptibility related with the ESRRβ polymorphism.

Results: A multiple regression analysis showed that individuals with rs61742642 CT genotype showed greater ATTS (β= 10.498 dB, CI = 6.413 – 14.583, p <0.001) without convincing evidence of change in DPTLS (β = -0.037 dB, CI = -0.663 – 0.589, p = 0.906), TETLS (β = -0.467 dB, CI = -1.573 – 0.640, p = 0.401) and TETSS (β = 0.224 dB, CI = -0.111 – 0.559, p = 0.186) compared with individuals with rs61742642 CC genotype. Individuals with the CT genotype showed poorer pre-exposure audiometric thresholds from 3 to 6 kHz in both ears with compromised DPOAE amplitude (β = -1.409 dB, CI = -2.662 – -0.156, p = 0.028) and TEOAE signal-to-noise ratio ($F(1, 53) = 5.23$, p = 0.026) in left ear. TEOAE 1/3 octave signal-to-noise ratios were higher ($F(1, 53) = 5.037$, p = 0.029) for females compared to males.

Conclusion: The results indicate that individuals with the CT genotype are likely to get greater amount of metabolic compromise in cochlear physiology compared with individuals carrying CC genotype. The study associated the rs61742642 CT genotype with compromised pre-exposure poorer audiometric thresholds, reduced DPOAE amplitude and compromised TEOAE signal-to-noise ratio compared to individuals with CC genotype. The study suggests that the ESRRβ polymorphism is associated with

increased susceptibility to NIHL, and also indicates the efficacy of otoacoustic emissions testing for identifying sound processing endophenotypes.

A POLYMORPHISM IN HUMAN ESTROGEN-RELATED RECEPTOR BETA (ESRRβ) IS ASSOCIATED WITH PHYSIOLOGICAL MEASURES OF NOISE-INDUCED HEARING LOSS

by

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APPROVAL PAGE

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
ATTS	Audiometric Temporary Threshold Shift
BM	Basilar Membrane
DBD	DNA Binding Domain
DPOAE	Distortion Product of Otoacoustic Emissions
DPTLS	DPOAE Temporary Level Shift
ER	Estrogen Receptor
ERR	Estrogen-Related Receptors
ESRRβ	Estrogen-Related Receptor Beta
GR	Glucocorticoid Receptor
IHC	Inner Hair Cells
LBD	Ligand Binding Domain
NHR	Nuclear Hormone Receptor
NIHL	Noise Induced Hearing Loss
NR	Nuclear Receptor
OAE	Otoacoustic Emissions
OHC	Outer Hair Cells
ONR	Orphan Nuclear Receptor
PTS	Permanent Threshold Shift
ROS	Reactive Oxygen Species
SV	Stria Vascularis

TEOAE	Transient Evoked Otoacoustic Emissions
TETLS	TEOAE Temporary Level Shift
TETSS	TEOAE Temporary Suppression Shift
TTS	Temporary Threshold Shift

CHAPTER I

INTRODUCTION

Auditory system perceives intricate aspects of sound waves, processes incoming acoustic information and decodes their underlying communicative intent through a range of parallel and sequential processes (Näätänen, 1990). The cochlea is an end organ of peripheral auditory system which is responsible for frequency specific mechanical-chemical-electrical transduction (Liberman, 1982). Frequency specificity is primarily achieved by the distinct mechanical properties of the basilar membrane (BM). The BM is a sensitive cochlear epithelium responsible for the transduction process. The BM stiffness gradient makes it responsive to high and low frequencies at base and apex respectively (Johnstone, Patuzzi, & Yates, 1986). The tonotopic map on the BM is thought to be like a piano. It contains systematically organized rows of sensory and supporting cells. There are two types of sensory cells present in the cochlea: (1) outer hair cells (OHCs) and (2) inner hair cells (IHCs). OHCs are primarily responsible for non-linear cochlear amplification (Ruggero, Rich, & Recio, 1996). Cochlear amplification is a term used to describe active (i.e. energy drive) outer hair cell responses to sound, leading to higher sensitivity of the auditory system to lower intensity sounds (below 60 dB SPL) (Brownell, 1990). OHCs and IHCs have chemico-electrical transduction channels to convert sound pressures into chemico-electrical impulses (Liberman & Beil, 1979). These impulses are processed by the central auditory pathways and auditory cortices to extract

an underlying communicative intent (Tchorz & Kollmeier, 1999). Research on auditory physiology has demonstrated that it is vulnerable to noise-induced damage (Lamm, 1996) and the damage is manifested clinically as Noise-Induced Hearing Loss (NIHL).

NIHL is usually defined by audiometric hearing loss at the frequency range between 3 to 6 kHz accompanied by history of noise/music exposure. It is the second most common form of permanent hearing loss affecting all age groups (NIDCD, 2008, 2010). An estimated 12.5% of American children and 15% of American adults have permanent NIHL due to excessive acoustic exposure during work or in leisure activity (NIDCD, 2010). Professionals like factory workers, soldiers and musicians are exposed to loud sounds on a daily basis because of the occupational needs. Outside of the work environment, many individuals are exposed to loud sounds during recreational activities – like listening to MP3 player music, attending dance clubs and concerts, etc (Clark, 1991). Recent literature indicates that approximately 48% musicians are exposed to sound pressure levels exceeding National Institution for Occupational Safety and Health standards on a regular basis (Phillips et al., 2008) and an estimated 45 % of college-age musicians show early signs of NIHL in audiometric testing (Phillips et al., 2010). In the general population, the prevalence of NIHL increased from 9.2% to 18% from 1965 to 1994 in the United States, which was associated with a systematic increment in a routine acoustic exposure over the time duration (Wallhagen, Strawbridge, Cohen, & Kaplan, 1997). Mahboubi et al. (2013) studied National Health and Nutrition Examination Surveys (NHANES) data collected from 31,326 individuals aged 20 – 69 years across the United States from 1999 to 2004. They reported almost 12.8% prevalence of NIHL in

overall population. Literature signifies that NIHL is no longer limited to factory workers and musicians, but it has become a global hearing health concern.

NIHL is a complex disorder caused by a complex interaction between genetics and environmental factors. Complex disorders are generally defined as multiple factorial disorders as their causes are associated with multiple genes in combination with lifestyle and environmental factors (US National Library of Medicine, 2013). Complex disorders do not show a clear-cut pattern of inheritance and almost always show family clustering; this makes it difficult to determine a person's risk of inheriting or passing on these disorders (US National Library of Medicine, 2013). Therefore it is important to study gene-environment interaction in complex disorders like NIHL (Hunter, 2005). Environmental factors like noise exposure, music exposure and hours of daily music practice are not determining factor for NIHL (Phillips et al., 2008). Recent literature suggests that individual susceptibility plays an important role in acquiring NIHL (Konings et al., 2009a). There is no permanent cure for NIHL at present. Therefore, it is important to study genetic links to NIHL to identify genetically predisposed individuals well before they acquire permanent hearing loss. This line of research promises to reduce NIHL prevalence by facilitating personalized hearing healthcare planning.

CHAPTER II

REVIEW OF THE LITERATURE

Cochlear physiology is most vulnerable to noise-induced trauma (Lamm, 1996). The cochlea is situated in the petrous portion of the temporal bone. It is a snail shaped structure containing three ducts separated by two membranes. The scala vestibuli and scala media are separated by Reissner's membrane and the scala media and scala tympani are separated by the basilar membrane (BM) (Lim, 1980).

Noise-Induced Hearing Loss and Cochlear Physiology

The stria vascularis (SV) is a highly active metabolic area of the cochlea which provides nourishment to the vital cochlear structures. The SV is situated at the lateral wall and attached to the spiral ligament. Patuzzi (2011) suggested that the SV provides a mechanically distant nourishment source to the organ of Corti so that the mechanically active transduction process can take place in relatively less noisy area, and subsequently the mechanism can achieve better hearing sensitivity. The SV works as a metabolic power house to maintain the high concentration of K+ ions and positive extracellular potential (approximately + 80 mV) inside the scala media (Patuzzi, 2011).

K+ ions are physiologically one of the most important ions in the cochlea. Active circulation of K+ ions inside the cochlear structures is responsible for cochlear transduction. Endolymph shows a high concentration of K+ ions responsible for producing a high positive potential compared to intra- cellular fluid of the hair cells,

which shows a low concentration of K+ and high concentration of Cl- ions (Zidanic & Brownell, 1990). This creates a high-potential difference (almost 150 to 180 mV) between endolymphatic potential and intracellular fluid. A high electromotive force drives K+ ions into the hair cells when mechanical channels of the hair cell bundle are deflected away from their resting state by sound energy. These incoming K+ ions must be flushed out of the cell immediately to regain its original functional state. K+ ions can flow passively outside the cell membrane in the presence of electromotive force (Johnstone, Patuzzi, Syka, & Sykova, 1989; Spicer & Schulte, 1998). KCNQ4 channels situated at the basolateral membrane of the hair cells are described as major pathway for K+ ion exit. It has been proposed that the potential difference between endolymph and perilymph (scala tympani) may be a passive electromotive force which pushes K+ ions out of the cell and they are transported back to the intermediate cells of SV (Zdebik, Wangemann, & Jentsch, 2009). Fibrocytes and intermediate cells of SV receive the majority of incoming K+ ions from blood and regulate the positive potential inside the endolymph (Patuzzi, 2011).

The basilar membrane (BM) is a sensory epithelium of the organ of Corti which undergirds two types of receptor cells – outer hair cells (OHCs) and inner hair cells (IHCs) responsible for mechanical-chemical-electrical transduction (Küçük & Abe, 1989). The BM holds 3-4 rows of OHCs (n= 12,000 to 20, 000) and one row of IHCs (n= 3500) (Lim, 1980). 95% of afferent fibers from the spiral ganglion synapse at the IHCs with a hair cell to neuron ratio of 1.8:1 and the other 5% synapse to OHCs with a hair cell to neuron ratio of 5.7:1 (Hall, 2000, pp. 46). IHCs and OHCs synapse with type-I and II

ganglion neurons respectively. Auditory efferent fibers from the medial superior olive innervate OHCs and efferent fibers for lateral superior olive innervate IHCs (Hall, 2000, pp. 46, 47).

Greenwood (1961, 1990 &1991) and Stakhovskaya, Sridhar, Bonham, & Leake (2007) proposed a BM frequency map. Greenwood (1961, 1990 &1991) found that the BM is tonotopically organized. The BM shows a stiffness gradient across its length which makes it most responsive to high frequencies at the base and to low frequencies at the apex (von Békésy, 1960, p. 745). Gold (1948), Kemp (1978) and Davis (1983) argued that the remarkable dynamic range of human audition cannot be achieved without an active physiological amplification process inside the cochlea. This concept is referred as "cochlear amplification". Brownell, Bader, Bertrand, & de Ribaupierre (1985) observed that an isolated OHC becomes shorter with depolarization and longer with hyperpolarisation, and this unique property of OHCs was referred as "electromotility". The electromotility of OHC is driven by Prestin (i.e. a protein coded by SCL26A5 gene)-based molecular motor (Liberman et al., 2002). Ashmore et al. (2010) reviewed two major mechanisms underlying the cochlear amplifier theory – Prestin-driven somatic motility of OHCs and stereocilia-based active amplification processes. Ashmore et al. (2010) suggested that the electromotility of OHCs is a robust mechanism sufficient to inject power into the basilar membrane mechanics. Frank, Hemmert, & Gummer (1999) showed that segregated OHCs can vibrate faster than 1 kHz in response to electrical stimulation which makes it possible to amplify BM vibration in real-time. Verpy et al. (2008) studied the function of stereocilin – a protein connecting stereocilia to the tectorial

membrane in the cochlear mechanism - and found that the stereocilin knockout mice showed the absence of waveform distortions and suppression masking but present cochlear amplification. Therefore, it was inferred that the stereocilia bundle accounts for waveform distortion and frequency specificity of the BM, but not for the cochlear amplifier. Verpy et al. (2008) concluded that Prestin-driven OHC electromotility and functional integrity of stereocilia bundle are essential to maintain gain and frequency specificity of the cochlear amplifier respectively.

Cochlear hair cells transduce mechanical vibration of the BM into analogous electrical current, which further causes transmitter release onto associated spiral ganglion neurons whose axon projections connect with the central nervous system (Liberman, 1980). Usually, individual type II spiral ganglion neurons make a single presynaptic contact (i.e. ribbon) with the basolateral cell body of IHCs to receive transmitter release (Liberman, 1980; Kiang, Rho, Northrop, Liberman, & Ryugo, 1982 and Fuchs, 2005). Each inner hair cell possesses 10-30 synaptic ribbons, and it has been argued that the response phase of the ribbons (at the characteristic frequency of the nerve fibers) is independent of stimulus intensity which makes it possible to code time, intensity and frequency-related acoustic features of a stimulus (Fuchs, 2005). Pathophysiological changes in the spiral ganglion cells have been associated with audiometric hearing loss in individuals with auditory neuropathy (Trautwein, Sininger, & Nelson, 2000) and acoustic schwannoma (Glastonbury et al., 2002).

Cochlear homeostasis is a process to keep cochlear physiology in a "ready and steady" state to respond to acoustic stimuli which encompasses all aspects of cochlear

physiology except sensory transduction (Wangemann, 2008). It includes the process of energy production, maintenance of cations (K+, Na+, Ca2+ etc.) and anions (OH-, Cl- etc.), cell volume and pH of the cochlear fluids. Wangemann **(**2008) reviewed the literature on cochlear homeostasis and identified several homeostatic processes, which require constant gene and protein involvement. Below is the gist of the literature review by Wangemann **(**2008).

(1) Cell energy production: four genes of the SLC family (solute carrier family) have been identified in the cochlea responsible for glucose transport. SLC2A5 and SLC2A3 are localized near OHCs cell membrane and argued to transport glucose molecules actively inside the cells (Nakazawa, Spicer, & Schulte, 1995). SLC2A1 is expressed in SV cell layers and it transporters glucose to endolymph (Takeuchi & Ando, 1997). SLC16A1 is expressed in marginal cells of the SV and processes pyruvate and lactate to form ATP molecules (Okamura, Spicer, & Schulte, 2001).

(2) Free radicals, generated in a controlled amount, can serve as signaling molecules and are part of cellular redox homeostasis (Wangemann, 2008). It is argued that oxides and superoxides generated as by-products of the transduction process regulate a cascade of genetic regulations in the cochlea to maintaining cochlear homeostasis.

(3) It is important for the transduction process to maintain steep gradients of Na+, K+ and Cl- ions across the plasma membrane of hair cells and endolymph. K+/Na+/Cl- channels and related genes are responsible for maintaining the electrical gradients between cochlear fluids (Wangemann, 2008).

(4) Mutation in genes responsible to maintain cochlear homeostasis can lead to severe to profound deafness (Lukashkin et al., 2012).

Noise exposure can cause morphological and physiological changes in the cochlear structures, and different cochlear structures show different vulnerability to noise-induced damage (Cody & Robertson, 1983). It has been observed that frequent loud acoustic exposure can lead to permanent and/or temporary compromise of hearing sensitivity (Mills, 1973). Acoustic overload can cause (1) direct mechanical damage and (2) indirect chemical distress to the cochlear mechanisms (Hu, 2011). An acute noise exposure causes a temporary compromise in the auditory system that result in audiometric Temporary Threshold Shift (TTS). Chronic noise exposure (i.e. one time intense noise exposure – like a bomb blast, or repeated intense noise exposure over a period of time) can cause permanent compromise in the auditory system that result in audiometric Permanent Threshold Shift (PTS). It has been argued that the underlying mechanisms causing TTS and PTS are distinct (Henderson, Bielefeld, Harris & Hu, 2006).

Underlying Mechanisms for Temporary Threshold Shift (TTS)

Henderson et al., (2006) studied cellular mechanisms involved in TTS, and suggested that it is caused by direct mechanical injuries and indirect chemical distress to the delicate mechanics of the cochlea. Excessive vibration to the cochlear mechanism during noise exposure can cause direct mechanical trauma. Noise exposure can impose excessive vital demands on the mitochondria of cochlear cells and result in increased reactive oxygen species (ROS – negative oxygen ions) inside the cochlear cells, which

can cause secondary metabolic distress to the cochlear structures. Patuzzi (2002) studied the effects of intense tone exposure on the guinea pig cochlea. The investigator measured the cochlear microphonic (electrical potential produced by movement of stereocilia bundle during mechano-transduction) and the action potential of the auditory nerve using electrocochleography. The endolymphatic potential and intracellular potential were recorded using microelectrodes inside the cochlea. The endolymphatic potential was significantly increased, whereas intracellular potential, cochlear microphonic and action potential were significantly decreased following the noise exposure. TTS was attributed to the morphological deformities observed in the stereocilia bundle as the electrical gradient recorded between endolymph and intracellular fluid could not explain the decrement in neural threshold following noise exposure. Nordmann, Bohne, & Hearding (2000) studied the cellular basis of temporary threshold shift in animals exposed to intense octave band noise. Mechanical detachment of the stereocilia tip links from the tectorial membrane was observed in the electron microscopy following noise exposure. It was concluded that the decrements in the auditory nerve action potential and cochlear microphonic are a direct consequence of impairment in mechanico-electrical channels of the stereocilia bundle. Many investigators have further provided evidences suggesting that the mechanical damage to the stereocilia bundle is a primary mechanism leading to TTS (Jia, Yang, Guo, & David, 2009; Xiong, Wang, Yang, & Lai, 2013).

Wang, Hirose, & Liberman (2002) studied morphological changes in the stria vascularis in mice exposed to 94, 100, 106, 112 and 116 dB SPL noise. The compound action potential thresholds were recorded and the morphology of the stria vascularis was

studied just following noise exposures. The investigators found that 94 to 112 dB noise exposures for 24 hours cause TTS. The stria vascularis shows progressive morphological changes with respect to noise exposure. Exposure to 116 dB noise for 24 hours induced PTS and marked shrinkage of the strial cells. Hirose & Liberman (2003) studied endolymphatic potential and ABR thresholds in CaJ mice using the same method described by Wang, Hirose, & Liberman (2002). They found that 94 dB SPL caused 40 dB of TTS whereas 112 and 116 dB SPL caused >60 dB of PTS. TTS groups show degeneration of type II fibrocytes of the spiral ligament and strial edema at 24-hour post-exposure, but they did not find changes in endolymphatic potential. They showed that swelling and shrinkage of the stria vascularis disappears over a period of time and suggested that the reversible changes in the stria vascularis contribute to TTS.

Spoendlin (1971) studied 32 guinea pigs exposed to 100-138 dB SPL wideband noise for 1 minute to 1 hour and conducted electron microscopic examination of the auditory synaptic junctions. The investigator observed swollen auditory dendrites followed by a recovery phase over 48 hours after the noise exposures. Puel, Ruel, d'Aldin, C., & Pujol (1998) hypothesized that the synaptic damage observed following noise exposure is due to excessive release of glutamate inside the synaptic junctions by the IHCs. The hypothesis was tested by applying kynurenate - glutamate antagonist inside the cochlea following noise exposure. They found animals receiving kynurenate treatment showed a significantly lower threshold shift compared to the control group. Hakuba, Koga, Gyo, Usami, & Tanaka (2000) studied GLAST – glutamate transporter deficit mice and found that the GLAST-deficit mice showed increased accumulation of

glutamate inside perilymph and a higher compound action potential (CAP) threshold shift following noise exposure. The above evidence suggests that synaptic junctions of cochlear receptor cells are vulnerable to noise-induced mechanochemical distress leading to TTS.

Kujawa & Liberman (2009) reported that inner hair cell synaptic junctions are vulnerable to noise-induced damage. The investigators studied inner hair cell synaptic junction and neural density (using ribbon count approach) in basal ganglion following 2 hours noise exposure at 100 dB SPL in CBA/CaJ stain mice. They found that the noise exposure is sufficient to induce 40 – 50 dB TTS. ABR thresholds and the auditory nerve action potential return back to the baseline after 2 weeks of recovery. Distortion product of otoacoustic emissions (DPOAE) growth functions, which is a non-invasive testing of outer hair cell function, return back to baseline. However, supra-threshold ABR amplitudes do not return back to the baseline even after 8 weeks post-exposure. The investigators found significantly reduced density of IHC synaptic terminals and neurons at the basal ganglia. They concluded that even though action potential thresholds return back to the baseline, neural damage is evident as a complete supra-threshold ABR amplitude recovery is never achieved in laboratory animals showing TTS. Reduced supra-threshold ABR amplitude at peak V and peak I signifies that temporary threshold shift inducing noise has permanent effects on neural functioning. Lin, Furman, Kujawa, & Liberman (2011) reported a similar study replicating Kujawa & Liberman's (2009) finding. The investigators studied ABRs, DPOAEs and morphometry of IHC synaptic junctions. They induced TTS and PTS with 106 dB SPL and 109 dB SPL octave band

noise respectively. The investigators suggested that 2 hours of noise exposure at 106 dB SPL is somewhere nearer to the line of control which decides occurrence of TTS vs. PTS. The investigators found significant degeneration (followed by a small recovery) of IHC synaptic density. This evidence suggests that primary loss of inner hair cell synaptic fibers is an important sequel of the temporary threshold shift. However, there is no evidence that TTS-evoking laboratory exposures which do not cross the National Institute of Occupational Safety and Health (NIOSH) standards and widely used in the auditory research (Engdahl & Kemp, 1996; Marshall & Heller, 1998 and Attias, Sapir, Bresloff, Reshef-Haran, & Ising, 2004) shows the same type of sequel. Further research is needed to probe more into the molecular mechanisms responsible for TTS.

Recovery from Temporary Threshold Shift

Marshall & Heller (1998) studied recovery patterns of TTS using Bekesy audiometry and transient evoked otoacoustic emissions (TEOAE). They exposed participants to 105 dB SPL octave-band noise for10 minutes to evoke TTS. The recovery pattern for Bekesy audiometry and TEOAE showed a logarithmic function with time. This signifies that maximum recovery occurs just after noise exposure, and recovery gradually decreases with increase in post-exposure time. This finding suggests that underlying mechanisms for TTS (stereocilia bundle, stria vascularis and neural dendrites recovers) recover rapidly just after noise exposure, and their recovery become less rapid over a period of time. Similar findings were reported for DPOAE recovery function by Engdahl & Kemp (1996) and Sutton et al. (1994). TEOAE and DPOAE recovery functions are most sensitive to mechanical repair of hair cell bundle, OHC electromotility

and overall physiological state of OHCs (Hilger, Furness, & Wilson, 1995) whereas recovery function derived by audiometry is most sensitive to recovery from mechanical and chemical distress following noise exposure (Gates et al., 2002).

Sohmer & Pratt (1975) studied the temporary threshold shift in humans exposed to audiometric broadband noise at 90 dB SL (almost around 100 dB SPL) for 15 minutes. The auditory action potential was recorded over a period of 31 minutes post-exposure time. Rapid and gradual recoveries were observed in latency and amplitude of the action potential respectively. This finding suggests that even though nerve fibers achieve their spontaneous firing rate rapidly, neural synchrony takes more time to get restored.
It can be concluded that the first order neural recovery is very rapid and stabilizes very quickly, whereas recovery of cochlear hair cells, supporting cells and surrounding structures take comparatively more time. It takes a considerable amount of time to extract spiral ganglion cells from living organisms following noise exposure. Therefore, first order neural recovery is less understood. However, efforts have been made to study physiological changes in synaptic junctions and afferent neural dendrites following noise exposure. Ottersen et al. (1998) studied IHC synaptic junctions and observed an excessive amount of glutamate concentration in the synaptic junction immediately after noise exposure. Chen, Tseng, Liu, Lin-Shiau, & Hsu (2005) studied concentration of nitric oxide inside the stria vascularis and spiral ligament. They found almost 16.3 dB of TTS following 105 dB SPL noise for 10 minutes. Nitric oxide concentration was increased three-fold immediately after the exposure and decreased two-fold after 48 hours post-exposure time when auditory brainstem response thresholds returned back to

the baseline. After a week, nitric oxide concentration was found to be back to the pre-exposure level. The investigators suggested that increased amount of nitric oxide inside the cochlear neurons can interact with oxygen and form highly active peroxynitrite (ONOO-) ions. Excessive glutamate and nitric oxide (NO-) secretion inside the neural cells lead to neural excitotoxicity. Yamasoba, Pourbakht, Sakamoto, & Suzuki (2005) hypothesized that ebselen - a scavenger of peroxynitrite, can attenuate TTS by preventing NO- production inside the cochlear cells (especially inside the spiral ganglion cells). They found that TTS can be effectively limited by oral intake of ebselen, and the experimental group treated with ebselen showed less swollen dendrites compared to the control group following noise exposure. The above evidence highlights the role of reactive oxygen species like NO-, OHOO- etc. in the neural recovery process.

Effects of Repeated Noise Exposure on Temporary Threshold Shift

Canlon, Borg, & Flock (1988) proposed and tested an interesting analogy of cochlear exercise or training the cochlea to tolerate high levels of sound. According to the analogy, cochlear physiology has muscle-like properties. As muscles can be made by exercises to tolerate a high level of physical exertion, in the same way cochlear mechanics can also be trained to tolerate a high acoustic burden. The investigators exposed experimental animals to 81 dB SPL continuous tone for 24 days, whereas the control group was not exposed to any sound. Both groups were exposed to 105 dB SPL 1 kHz tone for 72 hours. ABR thresholds were measured after 90 minutes and 8 weeks following noise exposure. The animals not exposed to 81 dB SPL tone before traumatic noise exposure showed greater TTS and poorer recovery across the frequency range

compared to animals exposed to 81 dB SPL tone. It was concluded that somehow auditory physiology gets "tough" (thus referred as toughening effect) to noise-induced hearing loss after low level noise exposure before noise trauma.

Franklin, Lonsbury-Martin, Stagner, & Martin (1991) measured the time duration necessary to acquire predetermined DPOAE amplitude loss in order to study the effects of repeated non-traumatic noise exposures followed by a traumatic noisy event on OHC physiology. They found that animals exposed to repeated non-traumatic noise exposures take a longer time to acquire predetermined DPOAE amplitude loss compared to animals not exposed to repeated non-traumatic noise. It was concluded that OHCs physiology gets tougher after repeated non-traumatic sound exposures. Miyakita, Hellström, Frimanson, & Axelsson (1992) demonstrated a toughing effect in humans. The participants were exposed to 70 dBA music for 6 hours before TTS inducing noise exposure (105 dB SPL 2 kHz octave band noise for 10 minutes). Significant decrement in TTS at most of the audiometric frequencies was observed after the second week of music exposure. The toughing effect slowly diminished after the non-traumatic music exposure was removed. Campo, Subramaniam, & Henderson (1991) found that toughening effects were not only limited to TTS, but similarly affects PTS. It was hypothesized that the potential underlying mechanisms for conditioning effects might be: (1) middle ear muscle protection effect, (2) molecular substances generated by cochlear cells (like antioxidants, heat shock proteins etc.), and/or (3) medial superior olivary complex efferent fiber protection.

Ryan, Bennett, & Nigel (1994) studied effects of middle ear muscles on "toughening effects". They trained laboratory animals with non-traumatic stimuli to acquire toughening. They dissected middle ear muscles in a group of animals who have acquired a toughening effect. They compared post- middle ear muscles dissection effect on auditory toughening and found that middle ear muscles do not show a significant contribution to explaining the toughening effect. They concluded that the toughening effect is produced somewhere inside the cochlea.

Jacono et al. (1998) found that animals with a toughening effect show increased levels of glutathione reductase enzyme, y-glutamyl cysteine and catalase activity inside the stria vascularis. The investigators used "conditioning effect" for the phenomena to argue that the cochlear physiology is "conditioned" to produce higher pre-exposure concentrations of antioxidants inside the cochlea (which eventually reduce TTS and PTS) by repeated non-traumatic exposures. Antioxidants produced inside the cochlear cells (Jacono et al., 1998) or induced by extraneous agents (Kopke et al., 2005) significantly protect cochlear mechanics. This evidence suggests that production of antioxidants inside OHCs, IHCs, stria vascularis and spiral ganglia are primarily responsible for toughing/conditioning effects. Tanaka et al. (2009) demonstrated that this enriched acoustic environment following noise exposure significantly facilitates TTS recovery which was further associated with increased activity of antioxidants like glutathione (GSSG).

Attanasio et al. (1999) reported that dissection of the medial olivary complex significantly increases susceptibility to acquire TTS and PTS which makes it difficult to

assess a role of medial olivary complex on the toughening effect. However, it has been indicated by many investigators that efferent fibers play a role in acquiring a toughing effect (Zheng, Henderson, McFadden, & Hu, 1997; Kujawa & Liberman, 2009 and Attanasio et al., 1999).

There are many variables previously associated with TTS. Routine exposure to loud music and/or noise can induce conditioning effect and modify susceptibility to TTS (Miyakita, Hellström, Frimanson, & Axelsson, 1992 and Jacono et al., 1998). Individuals with brown eyes appear to be more resistant to TTS when compared to individuals with blue, green and hazel eyes (Hood, Poole, & Freedman, 1976). Smoking has been associated with increased susceptibility to acquire TTS (Ahn et al., 2011). It has been suggested that females are more susceptible to TTS than males (Dengerink, Dengerink, Swanson, Thompson, & Chermak, 1984). The variables associated with TTS listed above are controversial in hearing research. More research is needed to identify risk factors for TTS. A novel association to TTS has to be studied by controlling variables previously associated with TTS.

Underlying Mechanisms of Permanent Threshold Shift

The above-described mechanisms underlying TTS cause pathological changes in the cochlear transduction process and recover over a period of time. However, PTS is associated with a pathological permanent alteration of the cochlear physiology resulting from chronic single and/or multiple episodes of noise damage. More than three decades of research has systematically identified that the loss of cochlear hair cells is primarily associated with PTS. Henderson, Bielefeld, Harris & Hu (2006) suggested that reactive

oxygen species (ROS) and toxic free radicals are responsible for the hair cell death. Free radicals are molecules (e.g., superoxide, hydroxyl, peroxynitrite, hydrogen peroxide and ozone) with an unpaired electron, making them capable of altering the electron arrangements in the stable molecules. Campbell (2003) reported that ROS are highly unstable and short-lived molecules essential for cell life and molecular signaling, but they can trigger apoptotic and/or necrotic cell death if their concentration crosses a physiological threshold inside or in the surroundings of a cell. The underlying mechanism of hair cell death has been examined by applying chemical agents (like paraquat) with known ROS generation properties. Bielefeld, Hu, Harris, & Henderson (2005) used paraquat doses to generate ROS inside the cochlea. Paraquat was induced in the cochlea through round window and observed that a small and high paraquat doses can induce TTS and PTS respectively. The investigators reported that 10 mM paraquat dose induced hair cell death across the frequency range. ABR recorded on the 22nd post exposure day shows permanent hearing loss, high frequencies were more affected than lower frequencies and OHCs showed more vulnerability to paraquat-induced ROS than IHCs. It was concluded that ROS generation can lead to hair cell death and causes PTS.

Halliwell & Gutteridge (1999) reported the electron transport chain as one of the major mechanisms responsible for ROS production in the cochlea. It utilizes chemical energy from the ATP molecule to facilitate ion transportation between the cell membrane. The energy production process inside the mitochondria requires ATP molecules to interact with oxygen, and increased amounts of ATP and oxygen molecules are required to meet with the increased vital demands of an organism (Hoppeler,

Hudlicka, & Uhlmann, 1987). When mitochondria are using more oxygen to meet increased cellular demands for energy, more superoxide ions are generated as an unwanted byproduct (Henderson et al., 2006). Increased amounts of ROS inside the cellular environment produce a molecular signal to trigger cell death (Fleury, Mignotte, & Vayssière, 2002). Cell death can be caused by (1) apoptosis – an active (i.e. energy driven) process to eliminate non-functioning/unwanted cells from the organism, and (2) necrosis- a passive form of cell death caused by extraneous insult resulting in rupturing cell membrane and spilling out of intracellular contents (Hu, 2011). Both types of cell death are observed in the noise-exposed cochlea, and ROS and free radicals are the major underlying mechanism responsible for to cell death (Henderson, Bielefeld, Harris & Hu, 2006).

The effects of permanent NIHL on the auditory nervous system have been studied widely. Auditory nerve fibers show phase-locked spontaneous firing properties which are important to code the incoming acoustic signal (Palmer & Russell, 1986), and the temporal dynamics of auditory nerve fibers change with respect to their location on the basilar membrane (Hugh & Campbell, 1990). Scheidta, Kale, & Heinz (2010) studied the effects of these dynamic properties on the auditory nerve fibers in 10 normal hearing and 12 noise-induced hearing impaired chinchillas. The experimental group was exposed to 114 to 115 dB SPL 2 kHz octave band noise for 4 hours. The data collection was done 4 weeks post-exposure. The investigators reported poorer frequency selectivity, broadening of tuning curves and reduced action potential latency for the experimental animals with PTS. The investigators concluded that permanent NIHL adversely affects dynamic

properties of auditory nerve fibers which might contribute to poor speech perception ability reported in patients with NIHL (Carney, 1994). Kale & Heinz (2010) studied stimulus envelope coding abilities of auditory nerve fibers in chinchillas with permanent NIHL and it was observed that structure coding ability of the nerve fibers in permanent noise induced hearing loss did not change significantly in spite of broadening of tuning curves and increased neural thresholds. Heinz, Swaminathan, Boley, & Kale (2010) also studied across-fiber fine structure coding ability of the auditory nerve fibers in mice with permanent NIHL. They reported that as the travelling wave delay increases between two frequencies, neural cross correlation for fine structure decrease significantly. This evidence suggests that across-fiber coding mechanism is impaired in animals with PTS.

Pilati et al. (2012) studied the cellular physiology of the dorsal cochlear nucleus in mice with permanent NIHL. They found impaired synaptic transmission (especially inside the fusiform cells) accompanied with demyelination of neural axons of the dorsal cochlear nucleus. Aarnisalo, Pirvola, Liang, Miller, & Ylikoski (2000) found shrinkage of the neural cell body, the cell nucleus and increased incidence of apoptotic cell death in the nerve fiber of cochlear nucleus and superior olivary complex in animals with PTS. Gröschel, Götze, Ernst, & Basta (2010) demonstrated a histopathological difference between TTS and PTS in the auditory nervous system. They studied 11 mice exposed to 115 dB SPL broadband noise for 3 hours. 5 mice were sacrificed immediately after noise exposure and remaining 6 were sacrificed after 7 days post-noise exposure. The investigators found significantly reduced density of nerve fibers in ventral cochlear nucleolus for animals sacrificed immediately following the noise exposure (N= 5). Neural

density in the dorsal cochlear nucleus, inferior colliculus, medial geniculate body and 6 layers of primary auditory cortex was not significantly reduced in animals sacrificed immediately after the noise exposure, but it was significantly reduced for animals sacrificed 7 days after the noise exposure. The findings demonstrate that intense noise exposure accompanied with TTS produced immediate focal neural degeneration in the spiral ganglia and ventral cochlear nucleus, whereas permanent NIHL shows compromised neural density throughout the auditory nervous system. This evidence implies that permanent NIHL is not limited to the peripheral auditory system, but has profound consequences on the auditory nervous system.

Zhai et al. (2011) reported a potential treatment strategy to PTS by applying brain-derived neurotrophic factor into the cochlear. The neurotrophic factor shows nutritional effects on central and peripheral neurons, and it is necessary for survival of neurons following traumatic events (Song, Li & Han, 2008). Zhai et al. (2011) induced at least 75 dB SPL PTS before applying the neurotrophic factor. They found that animals treated with brain-derived neurotrophic factor showed lower PTS and higher neural density in the spiral ganglia compared to the control animals. It was concluded that application of brain-derived neurotrophic factor to the cochlear duct shows protective effects to noise-induced damage to the auditory nervous system.

The above literature suggests that outer hair cells, inner hair cells, synaptic junction of the hair cells, stria vascularis and neural cell body are vulnerable to noise induced damage. However, it has been shown that individual susceptibility to NIHL plays an important role in acquiring NIHL.

Genetic Links to Noise-Induced Hearing Loss

Intense noise exposure causes increased amounts of reactive oxygen species which leads to a temporary compromise of the metabolic and mechanical properties of the cochlea, such as the endolymphatic potential, the mechanoelectrical transduction channels of the stereocilia and synaptic junctions, resulting in temporary NIHL (Pattuzi, 2002 and Henderson et al., 2006). Chronic noise exposure triggers necrotic and apoptotic cell death or permanent NIHL (Bielefeld et al., 2005; Henderson et al., 2006 and Hu, 2011). Antioxidant treatments have been shown to effectively limit temporary (Yamasoba, Pourbakht, Sakamoto, & Suzuki, 2005) and permanent NIHL (Kopke et al., 2005) in animals. Though the exact underlying mechanisms and molecular pathways to mechanical and metabolic damage in temporary and permanent NIHL are not well understood (Nordman, Bohne, & Harding, 2000; Hu, 2011), it has been shown that direct mechanical trauma to the basilar membrane and organ of Corti can lead to mechanical disruption of hair cell body, stereocilia bundle, cuticular plates and supporting cells; it also causes direct hair cell lesions (Hu, 2011). Mechanical trauma to the cochlear structures can overdrive pathways responsible for maintaining cochlear homeostasis (Yamasoba, Pourbakht, Sakamoto, & Suzuki, 2005; Wangemann, 2008). This results in increased amount of reactive oxygen species inside the cell (Henderson et al., 2006) and if the self-defense mechanism maintaining cochlear homeostasis cannot overcome this excitotoxicity, the reactive oxygen species may trigger apoptotic or necrotic hair cell death (Ohinata, Miller, & Schacht, 2003) and lead to permanent NIHL. These molecular pathways leading to cell death are influenced by gene-environment interaction (Van Laer

et al., 2006 and Pawelczyk et al., 2009). It has been reported consistently in that some individuals are susceptible to NIHL when compared with others (Van Laer et al., 2006; Yang et al., 2006; and Pawelczyk et al., 2009). It is hypothesized that genetic variability between individuals is a major underlying mechanism influencing NIHL susceptibility.

Gene-Environment Association Studies of Noise-Induced Hearing Loss

Most of the studies reported in the literature compared genetic variability between the 10% most susceptible (i.e. showing most audiometric hearing loss) and the 10% most resistant (i.e. showing least audiometric hearing loss) individuals after statistically controlling the effects of age and noise exposure on audiometric hearing loss (Van Laer et al., 2006; Yang et al., 2006; Sliwinska-Kowalska, Noben-Trauth, Pawelczyk, & Kowalski, 2008; Pawelczyk et al., 2009; Konings et al., 2009a,b). Van Laer et al., (2006) studied 35 single nucleotide polymorphisms (SNPs) from genes involved in K+ ion circulation inside the cochlea. SNPs are defined as a type of polymorphism involving the variation of a single base pair (National Human Genome Research Institute, 2013). Van Laer et al., (2006) found a significant association for KCNE1 and KCNQ4 SNPs with NIHL. They verified the functional significance of the genetic association using the Chinese hamster ovary cell model and demonstrated that the cells with KCNE1 and KCNQ4 variants operate potassium channels at lower voltage levels compared to cells with wild-type KCNE1 and KCNQ4 variants. Pawelczyk et al. (2009) studied Polish noise-exposed factory workers and replicated the KCNQ4 SNP associated with NIHL in Van Laer et al. (2006).

Oxidative stress is a major contributing mechanism leading to activate apoptotic and necrotic pathways following noise exposure (Gechev, Van Breusegem, Stone, Denev, & Laloi, 2006 and Henderson et al., 2006). Furtunato et al. (2004) reported SNPs in PON2 and SOD2 genes associated to NIHL. Carlsson et al., (2005) studied SNPs in GSTM1, CAT, SOD, GPX, GSR and GSTP1 genes and found no significant association with NIHL. They attributed this null finding with the small sample size. Konings et al. (2007) reported 2 SNPs in CAT gene showing significant interaction effect with noise exposure. The interaction values for both SNPs were significant for two independent industrial populations. Lin et al. (2009) reported 2 SNPs in SOD2 genes associated with NIHL, one of these SNPs was previously associated to NIHL in Furtunato et al. (2004). GSTM1 rs10549055 nucleotide deletion was identified and replicated in independent NIHL populations (Lin et al., 2010, and Shen et al., 2012). SNPs in GSTP1 and GSTT1 are associated with NIHL in independent NIHL populations (Lin et al., 2010), but fail to replicated by Shen et al. (2012).

Yang et al. (2006) reported that NIHL susceptibility is associated with the hsp-70 genes, a family of genes responsible for producing heat shock proteins that prevent apoptotic cell death by activating the endoplasmic reticulum stress sensor protein against stressors like heat, noise and ototoxic drugs in noise-exposed factory workers. They found that an hsp-70 variant haplotype is associated with NIHL susceptibility. Konings et al. (2009b) studied two independent industrial populations to associate SNPs in HSP70 gene with NIHL. They reported a SNP in HSP70 associated with NIHL in both independent industrial populations. Two other SNPs in HSP70 showed association to

NIHL in only one population and fail to get replicated in the other. Konings et al. (2009b) concluded that genetic variability in HSP70 is associated with NIHL.

Sliwinska-Kowalska et al. (2008) reported that Cadherin 23, a gene expressed in intra-stereocilia links is associated with NIHL susceptibility. Konings et al. (2009a) studied 644 SNPs for 53 cochlear genes in two independent factory worker populations. They reported that protocadherin 15 and MYH14 are significantly associated with NIHL susceptibility in both populations. Liu et al. (2010) studied CuZu-superoxide dismutase-SOD1, an enzyme responsible for removing oxides and superoxide from the cell environment to prevent hair cell death. They found that the SOD1 variant was associated with a protective effect against NIHL. Lin et al. (2009) studied the effects of glutathione, a cellular antioxidant, S-transferase M1, T1 and P1 polymorphism on temporary NIHL (i.e. temporary threshold shift measured using the conventional audiometry) and found that glutathione S-transferase null individuals showed a higher amount of temporary NIHL than others. Lin et al. (2010) examined the effects of N-Acetyl-cysteine (a drug replenishing glutathione), intake on temporary NIHL (i.e. temporary threshold shift evaluated using the conventional audiometry), and found that the magnitude of temporary NIHL was significantly reduced for a group receiving the N-Acetyl-cysteine compared to the placebo taking group. The above evidence highlights molecular basis of NIHL susceptibility. Table 1 shows summary of human gene-environment association studies on NIHL.

Phillips et al. (2012) studied genetic associations to NIHL in music students aged 18-25 years to limit age-related confounding variables found in factory worker

population. They measured hearing sensitivity from 1 kHz to 8 kHz using conventional audiometry and defined an audiometric 4-6 kHz notch phenotype. The audiometric 4-6 kHz notch was defined as a successive drop in hearing threshold at 4-6 kHz compared with preceding lower frequencies followed by at least 5 dB of recovery at 8 kHz. The investigators used the 4-6 kHz audiometric notch configuration opposed to audiometric threshold at 3 kHz or 4 kHz (Konings, et. al, 2009a,b; Pawaleczyk et al., 2009; Lui et al., 2010) because it has been shown that music causes hearing loss around the 6 kHz region opposed to industrial noise which causes hearing loss around 3 to 4 kHz region. They found that a SNP (rs61742642) in Estrogen-Related Receptor Beta (ESRRβ) was associated with susceptibility to NIHL. They did not find statistically significant associations to NIHL with age, gender and noise exposure history unlike the other studies. Their results indicate that genetic variability related with ESRRβ is associated with susceptibility to NIHL. ESRRβ is a nuclear receptor essential for maintaining cochlear physiology (Collin et al., 2008).

Table 1. Summary of Human Gene-Environment Association Studies on NIHL

Genes	Single Nucleotide Polymorphism (SNP)	Replicated in Independent populations		References
		Only in one study	In more than one studies	
	Potassium Ions recycling genes			
KCNE1	rs2070358	Yes	Yes	Van Laer et al. (2006)
	rs1805127			
	rs1805128			Pawelczyk et al. (2009)
KCNQ1	rs163171			
	rs231899 (Interaction)			Konings et al. (2009a)
	rs2056892 (Interaction)			
	rs11022922(Interaction)			Sliwinska-Kowalska & Pawelczyk (2013)
	rs463924 (Interaction)			
	rs7945327 (Interaction)			
	rs718579 (Interaction)			
	rs2283205 (Interaction)			
KCNQ4	p.H.455Q	Yes		
GJB2	rs3751358			
	M34T (Interaction)			
GJB1	rs1997625 (Interaction)			
GJB4	rs755931 (Interaction)			
KCNJ10	rs1130183 (Interaction)			
KCNMA1	Rs1436089			
	Oxidative Stress Genes			
CAT	rs564250 (Interaction)			Fortunato et al. (2004)
	rs1001179 (Interaction)			
	rs494024 (Interaction)	Yes		Shen et al. (2012)
	rs12273124(Interaction)			Konings et al. (2007)
	rs475043 (Interaction)	Yes		Lin et al. (2009)
GSTM1	rs10712361	Yes	Yes	Lin et al. (2010)
GSTP1	rs1695			Sliwinska-Kowalska & Pawelczyk (2013)
GSTT1	rs10549055			
PON2	S311C			
SOD2	IVS3-23T/G	Yes		
	IVS3-60T/G			
	Heat Shock Protein Genes			
HSP70	rs1043618			Konings et al. (2009b)
	rs1061581			
	rs2227956	Yes		Yang et al. (2006)
				Sliwinska-Kowalska

			& Pawelczyk (2013)
	Monogenic Deafness Genes		
CHD23	rs11592462		Sliwinska-Kowalska,
GRHL2	rs1981361		Noben-Trauth,
ITGA8	rs10508489		Pawelczyk, &
KCNMA1	rs1436089 (Interaction)	Yes	Kowalski (2008),
MYH14	rs667907 (Interaction)	Yes	Konings et al.
	rs588035 (Interaction)	Yes	(2009a),
PCDH15	rs7095441	Yes	Phillips et al. (2012)
	rs11004085		Sliwinska-Kowalska
POU4F3	rs891969 (Interaction)		& Pawelczyk (2013)
ESRRβ	rs61742642		

Note: (Interaction) suggests interaction between SNP and noise exposure is associated with NIHL

Nuclear Receptors (NRs) and Cochlear Physiology: Toward a Theoretical Model of ESRRβ rs61742642 Single Nucleotide Polymorphism-related Susceptibility to NIHL

NRs are a morphologically related but diverse array of transcriptional factors that regulate homeostasis, reproduction, development and metabolism (Robinson-Rechavi, Garcia, & Laudet, 2003). The NR superfamily includes Nuclear Hormonal Receptors (NHRs), for which the activating ligand is known, and Orphan Nuclear Receptors (ONRs), for which they are not. It has been shown that lipophilic hormones can traverse the plasma membrane to the cell interiors and activate NHRs by binding with their ligand binding domain to transduce a biological signal from glucocorticoids, mineralocorticoids, the set steroids (like estrogen, progesterone, and androgen), thyroid hormones and vitamin D (Olefsky, 2001). ONRs are the transcriptional factors which appear morphologically similar to NHRs. ONRs are named so because their ligands were unknown at least at the time while they were discovered (Olefsky, 2001). The structural

organization of NHRs has been described in a review article by Kumar & Thompson (1999).

1. The N-terminal is a first part of the NHR. The N – terminal contains activation function 1 (AF1) which has been shown to weakly up-regulate transcription in the absence of a ligand (Wärnmark, Treuter, Wright, & Gustafsson, 2003).
2. The DNA-binding domain (DBD) is the most important part, which contains the most conserved amino acid sequence, meaning it has been conserved throughout species evolutionarily, which implies great importance. It contains two zinc–finger motifs with highly conserved cysteine molecules coordinating with binding of the zinc molecule. These zinc fingers can interact with a specific DNA sequence and regulate transcription of a specific group of genes (Kumar & Thompson, 1999).
3. The Hinge region is a flexible portion of the NR molecule which physically connects the DBD and LDB (Greena & Chambon, 1988)
4. A ligand binds to a Ligand Binding Domain (LBD) and makes NHRs active. The LBD contains a C-terminal which can sense the presence of a specific hormone or nonhormonal ligand in their environment (Olefsky, 2001). The exact LBD site may vary for different types of NHRs. The LBD also contains Activation Factor 2 (AF2) (Green & Chambon, 1988) which can be activated by a ligand and can modify the position of the AF2 activation helix which further regulates the transcription (Kumar & Thompson, 1999). AF1 activity is independent of AF2 activity (Glass & Rosenfeld, 2000). Some co-activators bind with AF2 which

synergizes with AF1 to robustly up-regulate transcription (McKenna, Lanz, & O'Malley, 1999).

5. The LBD can also bind with a co-activator or co-repressor to up-regulate or down-regulate transcription respectively (Kumar & Thompson, 1999).

Figure 1 shows how NRs can cause cascades of events inside the organism to regulate physiology. Glucocorticoid Receptor (GR) which might interact with ESRRβ (Munck & Guyre, 1984) is a good example for understanding how NHRs work at a cellular level. GR is ubiquitously present in almost all human tissues and it is a widely studied NHR (Munck & Guyre, 1984). The GR is activated by Glucocorticoids - a group of hormones secreted by the adrenal glands. The GR works as a hormone-activated transcription factor that regulates expression of the different genes responsive to Glucocorticoids (Kino, Charmandari, & George, 2010).

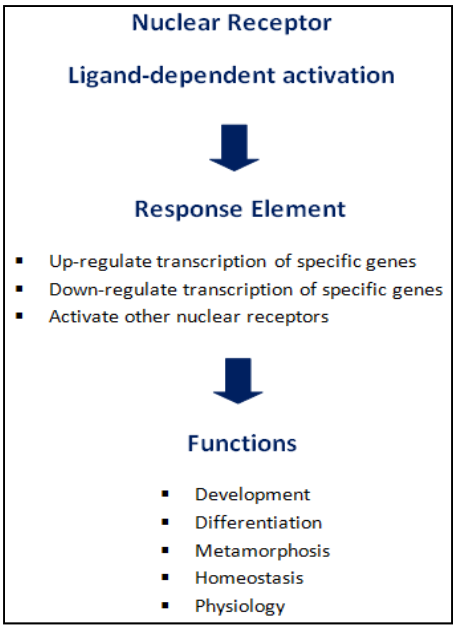

Figure 1. Cascade of Events Following Activation of NRs

Glucocorticoids were found to down-regulate the expression of GR mRNA which has been hypothesized to regulate transcription of specific DNA sequences known as Glucocorticoid Response Elements (Rosewicz et al., 1988). Galon et al. (2002) studied the effect of glucocorticoid on the gene expression profile of normal peripheral blood mononuclear cells. A DNA microarray analysis (classified into 3 categories – not regulated, up-regulated and down-regulated), quantitative TaqMan Polymerase Chain Reaction (PCR) and flow cytometry revealed that glucocorticoids simultaneously up-regulates and down-regulates anti-inflammatory soluble mediators and pro-inflammatory ligands respectively, this further explains its role in anti-inflammatory drug therapy. The study showed that 5 – 20 % of the genome can be classified as Glucocorticoid Response Elements – genes that can be activated by GR. The cascade of genetic events following

GR activation may explain beneficial and adverse effects of Glucocorticoid therapy on patients with endocrine problems.

NHRs are known as a ligand-dependent transcription factors which are activated by steroid hormones, thyroid hormones, retinoic acids or vitamin D (Robinson-Rechavi, Garcia, & Laudet, 2003). These transcription factors pass chemical signals from the cellular environment and regulate the cascade of cellular events to maintain physiology. A group of proteins has been identified which show structural similarities with NHRs, however, they are not regulated directly by the NHR activating ligands (hormones, retinoic acids or vitamin D) (Horard & Vanacker, 2003). There is no ligand identified for this group of transcription factors, and therefore, they are called Orphan Nuclear Receptors (ONRs). It is not yet clear whether these transcription factors work in a ligand-independent manner or if they get activated by some unknown ligands (Horard & Vanacker, 2003).

Giguère, Yang, Segui, & Evans (1988) identified Estrogen-Related Receptors (ERR) α and β from the structural similarity they shared with Estrogen Receptors (ER) α and β. ERRs are the first type of ONRs identified almost two and a half decades ago (Tremblay & Giguère, 2007). Chen, Zhou, Yang, & Sherman (2001) studied the morphology of the ERRs and ERs. They found structural symmetry between ERRs and ERs especially in their LBD. It was hypothesized that structurally similar ligands can bind both groups of the receptors. However, it has been shown that ligands activating ERs were not effective for activating ERRs (Horard & Vanacker, 2003 and Tremblay & Giguère, 2007). Morphological studies also show structural similarities in the DBD of

both groups of NRs. Lu, Kiriyama, Lee, & Giguere (2001) found that ERRs can regulate transcription of the estrogen-inducible pS2 gene - a regulatory target of ERs. Kraus, Ariazi, Farrell, & Mertz (2002) studied the estrogen response element transcription activity induced by ERRα and ERα in different cell types. It was observed that ERRα can work as a modulator of estrogen responsiveness, at the same time it can behave as an estrogen-independent activator. It was concluded that ERs and ERRs share a common portion of their response elements.

Efforts have been made to identify ligands for ONRs. Chen, Zhou, Yang, & Sherman (2001) studied the crystallographic structure of ERRs. Raghuram et al. (2007) provided indicative evidence for heme as a ligand for ERRα. ERRα is important for bone formation, bone remodeling, cellular proliferation and differentiation, whereas ERRβ is important for development, proper trophoblastic cell proliferation and differentiation. ESRRβ expression is essential in epithelial cells for endolymph production in the stria vascularis (Chen and Nathans, 2007) which is important to regulate ion homeostasis in the endolymph and organ of Corti (Wangemann, 2008). Raghuram et al., (2007) suggested that a ligand binding domain of ESRRβ shows structural similarities with other nuclear receptors that regulates cellular redox states. Collin et al. (2008) identified autosomal-recessive nonsyndromic hearing impairment due to a mutation in ESRRβ in a large consanguineous family of Turkish origin. Affected individuals of the family showed severe to profound sensorineural hearing loss with absent auditory brainstem responses and otoacoustic emissions. Their radiographic examination revealed normal morphology of the inner ear. Caloric testing revealed normal vestibular function and there was no

indication of visual, morphological or kidney related problems in the affected individuals. It was concluded that the autosomal-recessive hearing impairment was caused by a molecular disturbance in the cochlear physiology. Genome-wide fine mapping with micro-satellite markers revealed homozygous 7 bp duplication in exon 8. This gene sequence was identified as a coding sequence for the ESRRβ protein. Molecular modeling of the ESRRβ protein revealed 1 substitution in ESRRβ DBD and 3 substitutions in ESRRβ LBD. Molecular modeling showed that these mutations are sufficient to cause structural changes in the ESRRβ protein. The investigators studied the ESRRβ expression profile in the human inner ear. It was observed that ESRRβ is abundantly expressed in stria vascularis, basal ganglia, basilar membrane and spiral limbus, but it is not expressed in hair cells. Collin et al. (2008) concluded that the molecular changes in the ESRRβ protein specific to mutation in ESRRβ coding gene causes a subtle molecular break-down in the cochlear physiology resulting in severe to profound hearing loss. The investigators further suggested that ESRRβ might be important to mediate effects of thyroid, estrogen, glucocorticoid hormones which are important for development, homeostasis and physiology of cochlear structures. However, the specific mechanism underlying the ESRRβ mutation leading to profound hearing loss still remains a topic of further research.

Phillips et al. (2012) found that the ESRRβ rs61742642 polymorphism (CT) is associated with increased risk of acquiring NIHL (odds ratio = 2.7, p = 0.004) in music students aged 18-25 years. The rs61742642 C to T substitution replaces a proline with a serine in the ESRRβ amino acid sequence at P386S. This substitution resides in the

Ligand-Binding Domain of the receptor. The serine may cause the ligand-binding pocket to be less secure and subsequently leads to poor redox regulation in cochlear cells.

Appendix 1 shows a theoretical model proposed to explain how the ESRRβ rs61742642 variant can put an individual at increased risk to acquire NIHL. It can be observed that ERRβ rs61742642 is hypothesized to change morphology of ERRβ protein molecules, and the altered protein is not physiologically active enough to meet with increased demand put forward by noise exposure (such as redox cellular state) which manifested as higher magnitude and slower recovery to TTS. Chronic noise exposures can impose extra physiological demands on the cochlear physiology which cannot be well managed by individuals with the ESRRβ rs61742642 CT genotype. This induces poorly managed oxidative stress in the cochlea which subsequently leads to hair cell death and permanent NIHL.

Limitations of Gene-Environment Association Studies of NIHL and Toward a Model-Based Testing

It is important to note that the KCNQ1 variant rs2070358 (Van Laer et al., 2006) and GSTM1 rs10712361 are the only SNPs which have been consistently replicated in independent NIHL populations and showed physiological significance (Van Laer et al., 2006 and Pawelczyk et al., 2009, Shen et al., 2012). A KCNQ4 variant previously associated with NIHL susceptibility in Van Laer's study (2006) was found to be protective in Polish factory workers. Phillips et al. (2012) did not find significant associations for the same KCNE1 and KCNQ4 variants associated with NIHL in previous

studies (Van Laer et al., 2006 and Pawelczyk et al., 2009). Therefore, it is important to evaluate replication failures of NIHL genetic association studies.

Genetic links to NIHL susceptibility have been studied using a high frequency hearing loss phenotype evaluated by the conventional audiometry (Konings et al, 2009a,b; Pawaleczyk et al., 2009; Lui et al., 2010; Phillips et al., 2012). This approach has the following limitations:

- Outer hair cells (OHCs) are one of the most vulnerable cells to noise-induced excitotoxicity (Sha, Taylor, Forge, & Schacht, 2001), and it has been suggested that DPOAEs are more sensitive than conventional audiometry for detecting pre-synaptic noise-induced damage to OHCs (Atchariyasathian, Chayarpham, & Saekhow, 2008). However, OAEs are not utilized well in gene-environment association studies.
- Subjects must be exposed to loud environmental sounds before they show a measurable threshold shift in their audiogram. It is possible that genetically predisposed individuals might not acquire NIHL if amount of noise exposure is lower and/or the amount of resting period between exposures is longer. At the same time, it is possible that genetically non-predisposed individuals acquire NIHL because of a high amount of noise exposure and/or a low resting time between two exposures.
- It is hypothesized that genetic polymorphisms associated with NIHL susceptibility inhibit the cochlea from reinstating its original functional status following noise exposure (Henderson et al., 2006; Wangemann 2008) which

subsequently leads to audiometric hearing loss at 3-6 kHz (Van Laer et al., 2006, Konings et. al, 2009a; Pawaleczyk et al., 2009; Lui et al., 2010; Phillips et al., 2012). An NIHL phenotype defined by audiometric thresholds before and after noise exposure over a course of time has advantages as an indicator of changes in the auditory physiology pertaining to noise exposure compared with NIHL phenotype defined by baseline audiometry.

Permanent NIHL cannot be produced in humans in a controlled laboratory situation because of the ethical concerns. Therefore, many investigators have studied temporary/reversible NIHL in humans (Engdahl & Kemp, 1996 and Attias et al., 2004). TTS can be measured using a conventional audiometry and otoacoustic emissions (Engdahl & Kemp, 1996). It is reported that OAEs are more sensitive than conventional audiometry for detecting mechanical damage to the cochlea (Atchariyasathian, Chayarpham, & Saekhow, 2008). Therefore, OAEs can be helpful to explore genetic links to NIHL.

Otoacoustic emissions (OAE) as a Potential Tool to Explore Genetic Links to NIHL

Otoacoustic emissions (OAE) are the result of acoustic energy generated inside the cochlea, which is transmitted laterally through the middle ear ossicles and tympanic membrane (TM) into the external auditory canal (Kemp, 1978). This acoustic energy can be recorded by a sensitive microphone placed inside the closed external ear canal. Kemp (1978) first recorded click evoked otoacoustic emissions from the human ear canal and discussed its characteristics. OHC length decreases when it is depolarized and increases when it is hyperpolarized (Brownell, Bader, Bertrand, & de Ribaupierre, 1985).

Electromotility of the OHCs plays an important role in OAE production. Kemp (1986) suggested that a forward travelling wave is amplified by non-linear (or stimulus dependent) mechanical responses of the electromotile OHCs. Most of the energy generated by the OHCs amplifies vibration of the basilar membrane, but some fraction of energy escapes from the cochlea, primarily at the peak of the travelling wave where OHCs are highly active and reverted back to the ear canal. OHCs work as a re-emission source inside the cochlea and their motion creates backward travelling waves on the BM. Therefore, non-linearity induced by the electromotile OHCs are at the heart of the OAE production (Kemp, 1978 and 2002). OAEs have been classified by the different types of stimuli used to evoke them. Table 2 shows the conventional OAE classification (Kemp, 1978, 2002 and Hall, 2000). Transient Evoked Otoacoustic Emissions (TEOAE) is a term used to describe OAE recorded with transient stimuli like click and tonebursts. Usually the term "TEOAE" is inferred as click evoked OAE unless specified otherwise.

Table 2. Conventional Classification of Otoacoustic Emissions (OAEs)

Type	Subdivision	Description
Spontaneous OAE	----	Sound pressure is recorded from the ear canal in absence of external stimulation to the cochlea.
Evoked OAE	Click evoked OAE	Clicks are used to stimulate the basal portion of the cochlea. Forward and reverse cochlear travelling waves produced by the stimuli interact with one another and produce OAE responses with a wide frequency-intensity spectrum.
	Toneburst evoked OAE	Tone bursts produce mechanical distortion in the cochlear amplifier around their center frequency band and generate responses with a comparatively broad intensity-frequency spectrum (compared to DPOAE, SFOAE).
	Distortion Product OAE	These are evoked by the simultaneous presentation of two pure tones. The DPOAE has two distinct components (1) place-fixed component generated from 2f1-f2 distortion product frequency site on the BM, and (2) wave-fixed component generated from the site nearer to f2 where both f1 and f2 interact with each other.
	Single frequency OAE	This is evoked by single frequency stimuli. It is evoked by electromotile OHCs localized at a specific frequency region on the basilar membrane.

OAE as a Research Tool to Identify Cochlear Damage

Otoacoustic emissions are widely used as an objective tool to identify cochlear impairment in clinics. It is reported to be effective in screening newborns (Bray & Kemp, 1987), detecting noise-induced hearing loss (Attias, Horovitz, El-Hatib, & Nageris, 2001), monitoring ototoxic drug-induced early changes in cochlear physiology (Stavroulaki, Apostolopoulos, Seqas, Tsakanikos, & Adamopoulos 2001; Konrad-Martin, Reavis, Mcmillan, & Dille, 2012) and assessing patients with auditory neuropathy (Berg, Spitzer, Towers, Bartosiewicz, & Diamond, 2005).

Hotz, Probst, Harris, & Hauser (1993) found an 83% reduction in high frequency TEOAE amplitude level following military exercises. Attias, Horovitz, El-Hatib, & Nageris (2001) studied click evoked TEOAEs and DPOAEs for detecting and diagnosing patients with noise-induced hearing loss (NIHL). They assessed 283 noise-exposed subjects with hearing loss, 176 subjects with a history of noise-exposure without audiometric hearing loss and 310 young individuals with no history of noise-exposure and with normal hearing sensitivity. They found high sensitivity (79 to 95%) and high (84-87%) specificity for OAE testing for detecting individuals with noise-exposure. The investigators observed a reduction in DPOAE and TEOAE amplitudes in patients with NIHL and reported a systematic reduction of OAE amplitude for individuals with noise-exposure history without significant threshold shift in the conventional audiogram. Attias et al. (2004) studied temporary changes in the cochlear physiology following 10 minute exposure of 90 dB SL broadband noise in factory workers comparing oral magnesium intake vs. with placebo intake. They found that audiometric thresholds and DPOAE

thresholds (i.e. minimum intensity level at which DPOAE amplitude can be observed just above two standard deviations of the noise floor) shifted significantly less in the experimental group taking oral magnesium. It was concluded that the DPOAE data provides important information about the auditory physiology and complements audiometric TTS findings. Atchariyasathian, Chayarpham, & Saekhow (2008) studied 32 noise-exposed workers and 18 individuals without significant noise-exposure history. The investigators compared DPOAE results between groups: (1) ears with normal hearing and without significant history of noise-exposure, (2) ears with normal hearing and history of noise exposure and (3) ears with hearing loss and history of noise exposure. They found differences across the frequency range 250 Hz to 8 kHz for group 1 vs. 2 ($p < 0.0001$ for 4 k, 6 k and 8 k Hz) and 1 vs. 3 ($p < 0.0001$ across the frequency range). The above findings suggest that DPOAE testing is effective to identify mechanical damage in the cochlear mechanism induced by noise exposure.

Efforts have been made to predict hearing sensitivity/pure tone thresholds using the DPOAE input-output function. Boege & Janssen (2002) utilized optimized primary tone level settings ($L1 = 0.4L2 + 39$ dB) to measure DPOAE threshold. A linear regression model was used to predict L2 at which zero DPOAE amplitude can be elicited. The investigators found a moderate ($r = 0.64$, $p< 0.001$) correlation between DPOAE thresholds and audiometric thresholds. Hatzopoulos et al., (2009) utilized the optimized primary tone level setting technique used in Boege & Janssen (2002) and compared their findings with hearing thresholds and auditory steady state responses (ASSR) thresholds. The investigators found that DPOAE can predict hearing thresholds more accurately than

auditory steady state responses thresholds. Neely, Johnson, Kopun, Dierking, & Gorga (2009) studied the DPOAE input-output function and found that the technique is effective in assessing mild to moderate cochlear hearing loss. Recently Konrad-Martin, Reavis, McMillan, & Dille (2012) reported that a multivariate statistical model of a DPOAE ototoxicity monitoring paradigm produced satisfactory results in objectively identifying individuals with cisplatin-induced hearing loss. The investigators suggest that the same statistical model can be helpful to objectively identify individuals with a significant history of noise-exposure.

However, Shupak et al. (2007) reported that DPOAEs are not sensitive enough to be used as an objective assessment tool for NIHL screening. They studied 135 new factory workers and 100 subjects with no history of noise-exposure. They measured TEOAE, DPOAE and audiometric data from the subjects for 2 years and found that the DPOAE level shift after 2 years was not useful for predicting noise-exposure. They found higher sensitivity (86-88%) and lower specificity (33-35%) for TEOAEs compared to audiometry for predicting noise-exposure. The investigators concluded that OAEs cannot be used to objectively identify individuals with noise-exposure history. Hellman and Dreschler (2012) studied 233 participants with noise induced hearing loss and found large intra-subject variability with no clear relationship to DPOAE changes with audiometric hearing loss and concluded that clinical usefulness of DPOAE is limited because of high inter-subject variability. Additionally DPOAE and TEOAE show different sensitivity in various experimental protocols to detect individuals with noise exposure history (Hotz, Probst, Harris, & Hauser, 1993; Attias, Horovitz, El-Hatib, & Nageris, 2001;

Chayarpham, & Saekhow, 2008; Shupak et al., 2007 and Hellman and Dreschler, 2012). Audiometry, Spontaneous OAE, Single frequency OAE, DPOAE and TEOAE are affected differently by ototoxic agents (McFadden & Pasanen, 1994), noise exposure (Stavroulaki, Apostolopoulos, Segas, Tsakanikos, & Adamopoulos, 2001) and aging (Groh et al., 2006). Shera & Guinan (1999) argued that these observations cannot be explained if non-linearity of the cochlear amplifier is the only production mechanism for OAEs.

According to the conventional OAE production theory, mechanical distortion is induced by a forward travelling wave in the cochlear mechanics (Kemp, 1976, 1986). Additionally, the BM shows scaling symmetry (Rhode, 1971; Gummer, Smolders, & Klinke, 1987). The scaling symmetry implies that every frequency wave has to travel an equal number of cycles (or equal wavelengths) to reach their specific point on the BM (Shera & Guinan, 1999). Shera & Guinan (1999) hypothesized that the backward travelling wave re-emitted by OHCs needs to travel the same number of cycles (or wavelength) to reach the basal end of the cochlea. Therefore, it has been argued that the conventional non-linear distortion model of OAE production actually predicts phase independency of OAE signals over a wide range of frequencies (Shera & Guinan, 1999). Therefore, the conventional OAE production mechanism actually predicts constant phase delay (i.e. equal number of cycles are required for each frequency to travel back and forth to the cochlea) across the audible frequency range. Shera & Guinan (1999) showed that two separate components of distortion product of otoacoustic emission (DPOAE) have a distinct phase-frequency relationship. The two different components of DPOAE are: (1) a

distortion component, which is produced by a physical overlap of the two primary waves near the higher frequency region on the basilar membrane, and (2) a reflection component, which is produced by the cubic distortion (2f1-f2) region on the basilar membrane (Shera & Guinan, 1999). The investigators found that the distortion component maintains a constant phase delay across the frequency range and exactly follows the theoretical prediction. Their results imply that the mechanical non-linearity of the cochlea is a production mechanism for the distortion source. The reflection source does not show constant phase delay across the frequency range. Therefore, it was inferred that the mechanical non-linearity cannot be a production mechanism for the DPOAE reflection source. It has been observed that the single frequency OAE (Shera & Guinan, 1999; Kalluri & Shera, 2001) and transient evoked OAE (Kalluri & Shera, 2007) do not show constant phase delay across the frequency range which further suggests that mechanical non-linearity cannot be at the heart of their production mechanism.

On the basis of the above evidence, Shera (2004) described a mechanism-based taxonomy and its clinical utilities. Shera (2004) suggested distortion in the cochlear mechanics is primarily induced by the motion of the stereocilia bundle during the transduction process. Therefore, the distortion source of DPOAE is primarily sensitive to the stereocilia bundle. Shera (2004) suggested that the reflection component of DPOAE, SFOAE and TEOAE induced by low level stimuli are produced by preexisting irregularities in the cochlear mechanics like perturbation in OHC number across the cochlear length, geometric variations in OHCs and micromechanical perturbations within a cell body of OHCs etc. (Shera, 2004). DPOAE is more sensitive to changes in the

physiology of the stereocilia bundle whereas TEOAE induced by low intensity clicks is more sensitive to the changes in OHC cell bodies across a specific frequency band (Shera, 2004). DPOAE and TEOAE induced by loud stimuli are produced by a mixture of reflection and distortion components (Shera & Guinan, 1999) and can be used strategically to validate research findings. It is concluded that a comprehensive OAE test battery can be used to identify changes in different aspects of the cochlear mechanism. Gates, Mills, Nam, D'Agostino, & Rubel (2002) utilized DPOAE thresholds to differentiate mechanical vs. metabolic damage to the cochlea in patients with age-related hearing loss. Sudden decrement in the endolymphatic potential can more adversely affect audiometric hearing thresholds compared with DPOAE thresholds (Mills, Norton, & Rubel, 1993). Gates et al. (2002) hypothesized that: (1) a higher amount of audiometric threshold shift compared to DPOAE threshold shift is a sign of metabolic damage to the cochlea likely to be induced by stria vascularis damage, and (2) agreement between audiometric threshold shift and DPOAE threshold shift is a sign of mechanical damage to the cochlea likely to be induced by hair cell dysfunction. The investigators found that patients with age-related hearing loss show higher audiometric threshold shift and less DPOAE threshold shift which can be associated with strial atrophy (Mills, Schmiedt, & Kulish, 1990) and a subsequent decrement in the EP (Gratton, Schmiedt, & Schulte, 1996). The combination of audiometric threshold shift and DPOAE threshold shift can be an effective tool for differentiating metabolic/strial vs. mechanical/hair cell damage to the cochlear mechanism. However, this approach is not used in hearing research to explore genetic links to NIHL. A test battery combining audiometry and OAEs has not used to

develop different NIHL phenotypes to improve sensitivity and specificity of the gene-environment association studies of NIHL. It can be further helpful to test the model proposed in Appendix A.

Research Hypotheses

Hypothesis 1: Participants with the ESRRβ rs61742642 CT genotype would exhibit increased Audiometric Temporary Threshold Shift (ATTS) compared to participants with the ESRRβ CC genotype following the 10 minutes of 90 dB SL narrow-band noise exposure after statistically controlling for variables previously associated with temporary NIHL: gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile.

Rationale for Hypothesis 1: ESRRβ is important to regulate cellular redox state and maintain cochlear homeostasis; it is expressed in important cochlear structures such as spiral ganglion, supporting cells of the outer and inner hair cells, Reissner's membrane, stria Vascularis, and the spiral ligament. ESRRβ mutations lead to profound hearing loss (Collin et al., 2008). Phillips et al. (2013) found that musicians with the ESRRβ rs61742642 CT genotype show increased prevalence of bilateral notch phenotype compared to the individuals with the ESRRβ rs61742642 CC genotype (OR = 2.70, CI = 1.49 – 5.37, p = 0.0061). This implies that individuals with the CT genotype are more susceptible to NIHL. Therefore, it was hypothesized that the same amount of noise exposure (i.e. 90 dB SL for 10 minutes) would cause a greater amount of threshold shift in individuals with the CT genotype compared to individuals with the CC genotype.

Hypothesis 2: There would be no clinical difference between Distortion Product otoacoustic emission Temporary Level Shift (DPTLS) between participants with ESRRβ rs61742642 CT genotype compared to participants with ESRRβ CC genotype after statistically controlling for variables previously associated with temporary NIHL: gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile.

Hypothesis 3: There would be no clinical difference between overall TEOAE level shift (in dB) between participants with ESRRβ rs61742642 CT genotype compared to participants with ESRRβ CC genotype after statistically controlling for variables previously associated with temporary NIHL: gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile.

Rationale for Hypothesis 2 and 3: ESRRβ is not expressed in the cochlear hair cells (Collin et al., 2008). If the ESRRβ effect is autonomous (limited to cells showing ESRRβ expression), individuals with ESRRβ rs61742642 CT genotype will have compromised physiological function of Reissner's membrane, stria vascularis, supporting cells, spiral ganglion cells when compared to individuals with ESRRβ rs61742642 CC genotype, but both groups will show a similar compromise in the physiology of cochlear hair cells following the noise exposure.

It is hypothesized that the ESRRβ CT variant alters the physiology of the stria vascularis and spiral ganglion following noise exposure which is manifested by a decrement in the gain of the cochlear amplifier (measured by audiometry) without

significantly changing its non-linearity (measured by TEOAE and DPOAE) compared to the ESRRβ CC variant.

There is evidence for a differential effect of metabolic vs. mechanical pathophysiology on audiometry and OAEs. For example, intravenous furosemide injection changes chloride concentration inside the endolymph and causes a sudden decrement in the endolymphatic potential (Rybak & Whitworth, 1986). It results in sudden drop of hearing thresholds in adult gerbils (Mills, Norton, & Rubel, 1993; Rübsamen, Mills, & Rubel, 1995). DPOAEs are less affected (DP/ABR threshold ratio is 1:3) compared to auditory brainstem responses (Mills, Norton, & Rubel, 1993) when the endolymphatic potential is decreased with furosemide injection. Decrease endolymphatic potential reduces gain of the cochlear amplifier more adversely than its non-linearity (Mills, Norton, & Rubel, 1993). Non-linearity in the cochlear mechanism is induced by mechanical properties of OHCs which is not directly affected with furosemide (Mills, & Rubel, 1995). Gates et al. (2002) found that DPOAEs were affected less compared to hearing thresholds in patients with age-related hearing loss. They attributed this observation to strial atrophy and subsequent metabolic changes inside the endolymph reported in previous studies on age-related hearing loss. A test battery including DPOAE and audiometry can be a non-invasive differential diagnostic tool to identify metabolic (i.e. endolymphatic and strial changes) vs. mechanical (i.e. basilar membrane) damage in the cochlea (Mills & Schmiedt, 2004). Patients with static and temperature fluctuating auditory neuropathy show better OAEs and cochlear microphonic compared to hearing thresholds and auditory brainstem responses (Starr et al., 1998). Elevated hearing

thresholds and normal otoacoustic emissions are consistent with pathophysiological changes in the spiral ganglion as well (Santarelli, Starr, Michalewski, & Arslan, 2008). Therefore, a test battery with audiometry and DPOAEs can be helpful to differentiate OHCs damage from strial and spiral ganglion damage following noise exposure.

Autonomous effect of the ESRRβ protein produced by the ESRRβ gene with rs61742642 CT variant is hypothesized (1) to produce significant deterioration in hearing sensitivity and (2) not to produce significant deterioration in OAEs (both TEOAE and DPOAE) compared to their CC counterparts following the noise exposure.

If the ESRRβ rs61742642 polymorphism has non-synonymous effect on cochlear physiology following the noise exposure, then the cochlear cells which are not showing ESRRβ expressions are likely to get affected by the noise exposure. In that case, it is expected that outer and inner hair cells which are not showing ESRRβ expression (Collin et al., 2008) get affected by the noise exposure significantly higher in individuals with ESRRβ CT genotype compared to CC genotype. This leads to mechanical, metabolic and neural damage to the cochlea which manifested as significant decrement in OAEs for individuals with ESRRβ CT genotype compared to individuals with ESRRβ CC genotype following noise exposure. Therefore, failure to provide statistical evidence for hypothesis 2 and/or 3 can be interpreted as non-synonymous effect of ESRRβ on cochlear physiology following noise exposure.

Hypothesis 4: Participants with the ESRRβ rs61742642 CT genotype would exhibit significantly reduced TEOAE temporary suppression shift (in dB) compared to participants with the ESRRβ CC genotype after statistically controlling for variables

previously associated with temporary NIHL: gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile.

Rationale for Hypothesis 4: Outer hair cell electro-motility is important for the generation of otoacoustic emissions, and is regulated by a prestin-based motor and acetylcholine-induced potassium ion out flow (Frolenkov, 2006). Nicotine α9α10 acetylcholine receptors are likely to regulate calcium dependent potassium ion channels which, in turn, are important in the regulation of OHC electro-motility (Frolenkov, 2006). Recent research suggests that estrogen receptors regulate expression of nicotine α9α10 acetylcholine receptors (Lee et al., 2011). ESRRβ may interact with the estrogen receptors (Collin et al., 2008) which might regulate expression of nicotine α9α10 acetylcholine receptors and modulate electro-motility of the outer hair cells. It is possible that a protein molecule created by the ESRRβ gene with rs61742642 CT genotype might not interact efficiently with the estrogen-receptors following noise exposure. This might result in delayed and/or impaired regulation of estrogen-receptors which, as noted above, have been shown to influence the concentration of nicotine α9α10 acetylcholine receptors in the outer hair cell synapse. Increased strength of the efferent suppression of outer hair cells is described as a protective mechanism to NIHL (Maison & Liberman, 2000). Therefore, it was hypothesized that individuals with the ESRRβ CT genotype will exhibit an inefficient ESRRβ protein which might poorly interact with the estrogen receptors and subsequently reduce the concentration of nicotine α9α10 acetylcholine receptors in the outer hair cell synaptic junction following noise exposure compared to individuals with efficient ESRRβ protein. Therefore, individuals with the ESRRβ CC genotype will show

higher concentration of nicotine α9α10 acetylcholine receptors in the synaptic junction following noise exposure (because higher TEOAE suppression is associated with better protection to NIHL) and acquire greater TEOAE temporary suppression shift compared with individuals carrying the ESRRβ CT genotype.

Above hypotheses were tested using a multiple linear regression model to statistically control potential effects of variables previously associated with NIHL. Smoking is a health-related habit consistently associated with temporary and permanent NIHL (Garabrant, Bernstein, & Krebsbach, 1987; Pouryaghoub, Mehrdad, & Mohammad, 2007 and Lin et al., 2009). Individuals with green or blue eyes have been associated with increased susceptible to NIHL (Da Costa, Castro, & Macedo, 2008). Repeated moderately intense music and/noise exposure can induce increase amount of antioxidants into the cochlea which subsequently leads to provide protective effect against NIHL (Miyakita, Hellström, Frimanson, & Axelsson, 1992). Gender differences (females are less susceptible than males mid frequency range) in NIHL susceptibility has been reported by few investigators (McFadden, Henselman, & Zheng, 1999 and Sliwinska-Kowalska et al., 2006). Therefore, effect of ESRRβ was evaluated by a linear regression model for statistically controlling effect of gender, smoking, eye color, recent acoustic exposure history, routine music exposure history and routine noise exposure history on temporary NIHL.

CHAPTER III

METHOD

The objective of the study was to test whether the ESRRβ rs61742642 polymorphism is associated with the physiologic measures of NIHL. Temporary NIHL was elicited by 10 minutes exposure to 90 dB SL audiometric narrow-band noise centered at 2 kHz in college age musicians. The rationale for the study is that audiometry and the OAE test battery have the sensitivity to identify different cochlear lesions, and combining these testing procedures can be helpful (1) to validate that ESRRβ rs61742642 CT is a predisposing genotype to NIHL as suggested by Phillips et al (2012), and (2) to differentiate underlying cochlear mechanisms for temporary NIHL related with the ESRRβ rs61742642 polymorphism.

Participants

Musicians with ages between 18 and 31 years were invited to participate in the study. A genetic database containing 271 single nucleotide polymorphisms (SNPs) for 52 cochlear genes was available to the current project. The database contains genotypes for 330 music students (45 and 276 individuals with the ESRRβ rs61742642 CT and CC variant respectively) from the University of North Carolina at Greensboro who took part in the R21 study exploring genetic basis to NIHL funded by NIH (R21DC009296-03) since August 2010. Multiple invitation emails were sent to these 330 participants and participants were recruited on a first-come first-served basis. 19 musicians (out of 45)

with ESRRβ rs6742642 CT genotype and 40 musicians (out of 276) with ESRRβ rs6742642 CC genotype were recruited from the UNCG music student population.

Inclusion Criteria

1. Pure tone hearing thresholds at 1, 2, 3, 4, 6 and 8 kHz were $\leq$ 25 dB HL.
2. Normal otoscopic examination
3. Middle ear examination (immittance audiometry) within normal limits. Normal limits were defined as type "A" tympanogram (+/- 70 daPa middle ear pressure, 0.33 cc > middle ear compliance < 1.75 cc and 0.8 cm3 > ear canal volume < 1.8 cm3).
4. At least 2 out of 3 frequencies (500 Hz, 1 kHz and 2 kHz) must have present acoustic reflexes (≤ 105 dB HL)

Exclusion Criteria

1. Participants with pure tone thresholds higher than 25 dB HL at audiometric frequencies 1, 2, 3, 4, 6 and 8 kHz were excluded from the study.
2. Participants with complaint and/or history of active ear infection, chronic tinnitus, ear disorders such as otosclerosis, ossicular chain dislocation, immunological disorders and neurological disorders were excluded from the study.
3. Participants with abnormal immittance audiometry results were excluded from the study.
4. Participants with a 2 kHz narrow-band noise threshold above 10 dB HL were excluded.

Data Collection Procedure

The study was approved by the Institution Review Board, the University of North Carolina at Greensboro (11-0335). An informed consent was obtained from participants before admitting them into the study. The participants were requested not to expose to loud sounds such as lawn mowers, vacuum cleaners, motor bikes, MP3 player music, heavy rock music etc. for at least 14 hours before testing. The data was collected in a sound treated booth meeting ANSI standards in the School of Music, UNCG.

Pre-requisite testing: Conventional audiometry was performed to measure hearing thresholds for pure tones at 1, 2, 3, 4, 6, 8 kHz and narrow-band noise centered around 2 kHz. The conventional audiometry was followed by immittance audiometry. Demographic information, family history, medical profile, cell phone usage profile, noise and music exposure profile were collected using an online survey built on qualtrics.com (Appendix A). The participants who met the inclusion criteria were selected for further testing.

Experimental Procedure: The participants were instructed to sit in an upright position on a chair for the entire study time. A calibrated AC-40 diagnostic audiometer with insert ER-3A receiver was used to measure hearing thresholds. A company calibrated ILO 292 USB– II (Otodynamics Ltd.) was used to record otoacoustic emission profile. All parameters including gain, filters, stimulus parameters, recording protocols etc. were calibrated and verified by Otodynamics. OAE probe calibration test recommended by Otodynamics. Ltd was run before testing each participant. Real ear ILO probe calibration

was performed using the ILO probe-fit check paradigm before running each OAE measurement.

The AC-40 audiometer was used to generate 90 dB SL narrow-band noise with a 2 kHz center frequency. The noise was delivered to the ear canal using a calibrated ER-3A receiver. Pre and post-exposure audiometry and OAE data were collected using the same acquisition parameters.

1. Audiometry: Pure tone thresholds were measured at 2, 3 and 4 kHz using the modified Hughson-Westlake procedure (in 5 dB steps) which defines hearing threshold as a minimum puretone intensity to elicit two out of three correct responses (Carhart & Jerger, 1959). Once threshold was obtained using this method, the pure tone intensity was increased to 5 dB SL and a 2 dB up – 1 dB down method was adapted to obtain more accurate hearing threshold. Pure tones were presented four times and the lowest stimulus intensity to elicit three correct responses was considered hearing threshold. Temporary threshold shift was calculated by subtracting pre-exposure hearing thresholds from post-exposure hearing thresholds at 2, 3 and 4 kHz respectively. A quantitative (i.e. area under the curve) Audiometric Temporary Threshold Shift (ATTS, in dB) was calculated by adding temporary threshold shift values at 2, 3 and 4 kHz together.
2. Distortion Product Otoacoustic emission (DPOAE): DPOAE input-output functions were measured to obtain DPOAE temporary level shift (DPTLS). DPOAE input-output functions were collected at 2, 3 and 4 kHz using f2/f1 = 1.22. Intensities of the primary tones (L1 and L2) were calculated using L1 =

(0.40) L2 +39 equation as it has been shown that a DPOAE input-output function elicited using unequal L1 and L2 results in a higher amplitude DPOAE input-output function and better estimation of cochlear physiology (Whitehead, McCoy, Lonsbury-Martin, & Martin, 1995; Kummer et al., 2000; Gorga, Neely, Dorn, & Hoover, 2003 and Hatazopoulos et al, 2008). DPOAE input-output functions were collected for L2 levels between 75 to 30 dB in 5 dB/octave steps (10 point resolution). DPOAE input-output function at 2, 3 and 4 kHz were collected. The DP amplitude data (in dB SPL) was converted into µPa using $p(in\ \mu pa) = 20(10)^{DP\ amplitude\ (in\ dB\ SPL)/20}$ (http://www.sengpielaudio.com/calculator-soundlevel.htm). Area under the curve was calculated by adding up all DP amplitude (in µpa) together, and multiplied the final figure by 10. Similar method was used by Gates et al. (2002). Pre-exposure DPOAE level was calculated by adding up pre-exposure DPOAE input-output function area at 2, 3 and 4 kHz. Post-exposure DPOAE level was calculated by adding up post-exposure DPOAE input-output function area at 2, 3 and 4 kHz. Pre-exposure DPOAE level (area in μpa^2) was converted in µpa by taking square root of μpa^2. The µpa data was converted into dB SPL using L (in dB SPL) = $20\log_{10}$ (µpa/20) (http://www.sengpielaudio.com/calculator-soundlevel.htm). Post-exposure DPOAE data were converted into dB SPL using the same method describe above. DPOAE Temporary Level Shift (DPTLS, in dB SPL) was calculated by subtracting post-exposure DPOAE level (in dB SPL) from the pre-exposure DPOAE level (in dB SPL).

3. Transient Evoked Otoacoustic Emissions (TEOAEs): TEOAEs were measured using ILO-292 fast screening protocol (12.5 msec window). TEOAEs were elicited by 84±3 peSPL non-linear sweep ensembles of 4 clicks, 1 of which is opposite in polarity and three times the amplitude of the other 3 clicks. The electric stimulus generated by ILO 292 is consisted of 80 μs rectangular pulses presented at the rate of 80 /sec. The noise rejection level was set at 6 mpascal, and the test was terminated after successful acquisition of 260 sweeps. TEOAE temporary level shift (TETLS, in dB) was measured by subtracting post-exposure TEOAE from the pre-exposure TEOAE amplitude (Marshall & Heller, 1998)
4. TEOAE contralateral suppression was elicited by 50 dB SL contralateral broadband noise. The broadband noise was delivered by the ILO-292 TEOAE probe. Pre-exposure TEOAE suppression was calculated by subtracting pre-exposure TEOAE amplitude with 50 dB SL contralateral broadband noise from pre-exposure TEOAE amplitude without contralateral noise. Post-exposure TEOAE suppression was calculated by subtracting post-exposure TEOAE amplitude with 50 dB SL contralateral broadband noise from post-exposure TEOAE amplitude without contralateral noise. TEOAE temporary suppression shift (TETSS, in dB) was calculated by subtracting post-exposure TEOAE suppression from pre-exposure TEOAE suppression.
5. Noise exposure: Audiometric narrowband noise centered at 2 kHz was presented for 10 minutes at 90 dB SL (90 dB above threshold). Post-exposure OAE and audiometry data was collected after a 2-minute recovery period following the

noise exposure. Kirk & Pattuzzi (1997) reported transient improvement in DPOAE amplitude just following exposure to low frequency tones for two minutes. The changes in active processes inside the cochlea are expected to improve DPOAE amplitude just after noise exposure. These changes are transient and rapid; therefore, they suggested evaluating cochlear physiology after 2 minutes of noise exposure to accurately measure DPTLS and ATTS following noise exposure. DPOAEs were collected first in 29 of participants (20 CCs and 9 CTs) and audiometry was performed first in rest of the participants (20 CCs and 10 CTs) following the noise exposure to balance recovery effect across audiometric and DPOAE testing. TEOAEs were recorded after audiometry and DPOAE testing.

ATTS, DPTLS, TETLS and TETSS were measured only in left ear because it has been shown that the left ear is more vulnerable to NIHL and left ear is used to explore genetic association to NIHL in previous studies (Van Laer et al., 2006; Konings et al., 2009a).

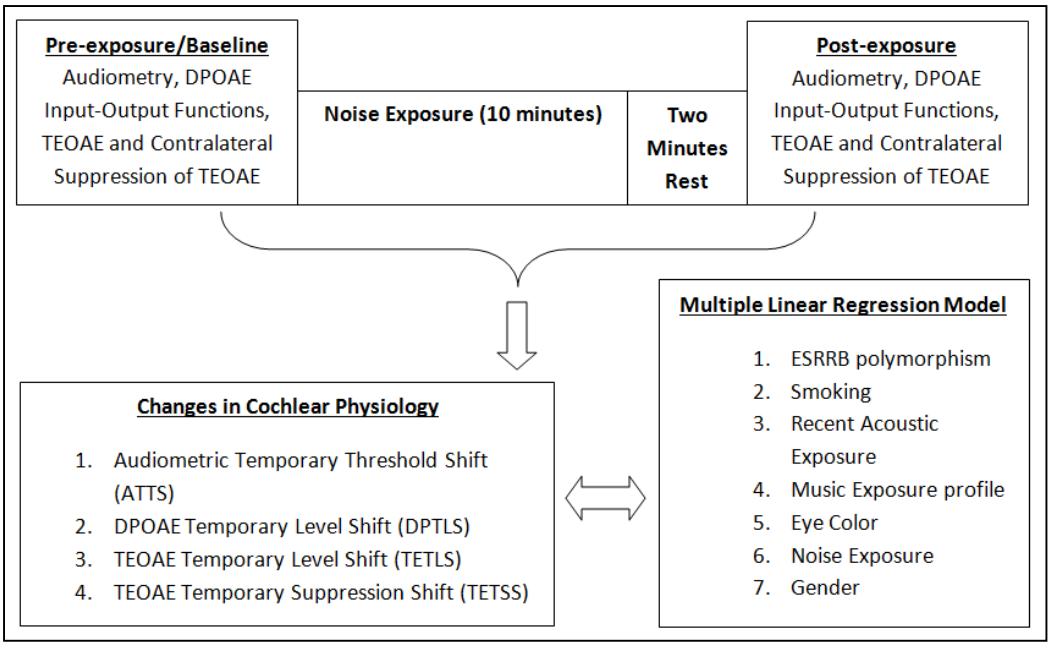

Figure 2. Schematic Diagram of Research Method

Data Analysis and Statistical Plan

All statistical analyses were performed using the IBM SPSS version 20 statistics package. A multiple linear regression model with 8 predictors – ESRRβ polymorphism, gender, smoking, recent acoustic exposure history, routine music exposure profile, routine noise exposure profile, eye color and audiometry/DPOAE testing counterbalance effect was used to test Hypotheses 1 (ATTS) and 2 (DPTLS). A multiple linear regression model with 7 predictors – ESRRB polymorphism, gender, smoking, recent acoustic exposure history, routine music exposure profile, routine noise exposure profile and eye color was used to test Hypotheses 3 (TETLS) and 4 (TETSS). TEOAEs were always collected after audiometry and DPOAEs, so the counterbalancing effect was not included for TETLS and TETSS analysis. Recent acoustic exposure history, routine

music exposure profile, routine noise exposure profile, eye color, smoking and gender were derived from the survey data. TEOAEs were collected always after audiometry and DPOAEs, so the counterbalancing effect was not included in the TEOAE analyses. ATTS and DPTLS were calculated by adding temporary threshold shift and temporary level shift at 2, 3 and 4 kHz.

Description of the Derived Variables used in the Multiple Linear Regression Analysis (derived from the Appendix B)

1. Smoking: Participants were asked to answer whether they smoke tobacco (cigarettes or cigars) on a regular basis. "Regular basis" was defined operationally as smoking greater than or equal to one cigarette/day for at least 3 months. The variable was coded binary: 1 – tobacco smoking on a regular basis and 2 – no tobacco smoking on a regular basis.
2. Gender: Female and male were coded as 1 and 2 respectively.
3. Recent acoustic exposure history: Participants were asked to report how many hours ago they were exposed to loud sounds such as music ensembles, practice sessions, MP3 player, FM radio, factory noise, heavy traffic, target shooting or vacuum cleaner noise etc. before the testing session. "Loud sound" was defined operationally as intense enough sounds in the environment to require an individual to raise one's voice to get heard by a person sitting nearby. The variable was coded in four categories, 1 – before 2 hours of testing, 2 – 2 to 12 hours before testing, 3 – 12 to 48 hours before testing and 4 – more than 48 hours before testing. The coding strategy was based on the TEOAE and Bekesy

audiometric recovery function reported by Marshall & Heller (1998). The investigators observed that maximum percentage of recovery in both measurements is accomplished within 1 to 2 hours of noise exposure and almost 100 % recovery in accomplished within 24 hours.

4. Music exposure: Participants were asked to provide information about their primary, secondary and tertiary musical instruments, the average time they spend practicing those instruments per week, music ensembles they were attending during the semester and time spent in each ensemble per week. Appendix C and D were used to rate musical instruments and ensembles. Primary, secondary and tertiary instrument scores (Appendix C) were added and multiplied by reported hours/week to calculate the instrument dependent music exposure scores (score A). Appendix D was used to rate music ensembles, and each music ensemble rating was multiplied by reported average time spent (hours/week) in each ensemble. The individual ensemble scores were added to calculate overall music ensemble rating (score B). Appendix C and D were developed on the basis of an expert opinion and noise dose measurement (unpublished work) for different ensembles at the school of music, UNCG, and some previous work by Phillips & Mace (2008), and Phillips, Shoemaker, Mace, & Hodges (2008). Participants were asked to provide information on how frequently they use personal music players on 5 point scale (1 = almost never, 2 = rarely, 3 = sometimes, 4 = most of the time, 5 = almost all the time during leisure), and how loud they use music device on a 100-point loudness scale. Music player exposure score was calculated

by multiplying these two variables (score C). Overall music exposure was calculated by adding score A, B and C. The quartile ranges were calculated and coded 1, 2, 3 and 4 for mild, moderate, moderately-severe and severe music exposures respectively.

5. Noise exposure: Participants were asked whether they were working at noisy places (where they need to raise their voice to get heard to a person sitting nearby), ride motorcycle, exposed to traffic noise on regular basis and whether they had ever been exposed to firearms. Participants reporting at least one incidence of routine work-related noise exposure was rated "1". Participants with a history of at least one incidence of firearm noise exposure were also rate "1" because it has been shown that a single incidence of impulse noise exposure is more adverse than repeated continuous noise exposures (Forget, 2011). Participants reporting routine and impulse noise exposures were rated "2".
6. Eye color: Participants were asked to choose their eye color. Participants with brown eye color were coded "1", and non-brown eye (i.e. green, blue and hazel) were coded "2".
7. Counterbalancing: To counterbalance recovery effect on audiometry and DPOAE, audiometry was followed by DPOAE for 29 participants (9 CTs and 20 CCs), and DPOAE was followed by audiometry for rest of the participants (10 CTs and 20 CCs) following 2 minutes of noise exposure. These incidences were coded "1" and "2" respectively. Similar methodology was utilized by Swanepoel & Hall (2010).

Research Hypotheses: Definition of Statistical Support

Hypothesis 1: Participants with the ESRRβ rs61742642 CT genotype would exhibit increased Audiometric Temporary Threshold Shift (ATTS) compared to participants with the ESRRβ CC genotype following the 10 minutes of 90 dB SL narrow-band noise exposure after statistically controlling for variables previously associated with temporary NIHL: gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile. Test-retest reliability of audiometry is 5 dB (Carhart & Jerger, 1959).

Definition of statistical support for Hypothesis 1: Test-retest reliability of audiometry is considered 5 dB for each frequency. A multiple linear regression analysis was performed on ATTS with 8 predictors. An unstandardized ESRRβ regression co-efficient with the lower confidence interval limit greater than 5 dB was defined as clinically significant difference between the ESRRβ groups.

Hypothesis 2: There would be no clinical difference between Distortion Product otoacoustic emission Temporary Level Shift (DPTLS) between participants with ESRRβ rs61742642 CT genotype compared to participants with ESRRβ CC genotype after statistically controlling for variables previously associated with temporary NIHL: gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile.

Definition of statistical support for Hypothesis 2: Test-retest reliability of DPOAE input-output function for multiple test probe setup ranges from 0.87 – 2.97 dB at 1 to 4 kHz (Wagner, Heppelmann, Vonthein, & Zenner, 2008). The lower limit of the 95%

confidence interval (i.e. 0.87 dB) was defined as meaningful difference between the ESRRβ groups. An unstandardized regression coefficient beta with confidence interval within ± 0.87 dB was considered statistical support for the hypothesis 2.

Hypothesis 3: There would be no clinical difference between TEOAE level shift (in dB) between participants with ESRRβ rs61742642 CT genotype compared to participants with ESRRβ CC genotype after statistically controlling for the variables previously associated with temporary NIHL: gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile.

Definition of statistical support for Hypothesis 3: Intra-subject test-retest reliability for broadband TEOAE response was reported 1.2 dB by Marshall & Heller (1996). Quaranta, Dicorato, Matera, D'Elia A, & Quaranta (2012) found 0.9 - 1.2 dB mean difference in TEOAE level shift (in dB) following 10 minutes of acoustic exposure between experimental groups. Therefore, 1.2 dB was defined as meaningful difference between the ESRRβ groups. An unstandardized regression coefficient beta with 95% confidence interval within ± 1.2 dB was considered statistical support for the hypothesis 3.

Hypothesis 4: Participants with the ESRRβ rs61742642 CT genotype would exhibit significantly reduced TEOAE temporary suppression shift (in dB) compared to participants with the ESRRβ CC genotype after statistically controlling for variables previously associated with temporary NIHL: gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile. Unlike audiometry, there are no acceptable test-retest criteria for the contralateral TEOAE suppression.

Definition of statistical support for Hypothesis 4: Recent research suggests that the minimum detectable TEOAE suppression shift ranges from 0.10 to 3.25 dB with a median of 0.91 dB and it is highly dependent on the signal-to-noise ratio obtained at frequencies below 4 kHz (Goodman, Mertes, Lewis, & Weissbeck, 2013). In this study, the lower limit of the detectable TEOAE suppression shift was considered appropriate to test hypothesis 4 as the effect of ESRRβ polymorphism on TETSS was expected to be small following a brief noise exposure. Therefore, an unstandardized ESRRβ regression co-efficient with the lower limit of the 95% confidence interval greater than 0.10 dB was considered clinically significant difference between the ESRRβ groups.

CHAPTER IV

RESULTS

Pre-exposure Audiometric and Otoacoustic Emission Findings

Descriptive statistics for the baseline puretone and immittance audiometric findings are shown in Tables 4 and 5 for participants with ESRRβ rs61742642 CC and CT allele genotypes respectively. Audiometric hearing thresholds were measured across the audiometric frequencies from 1 to 8 kHz using 5 dB steps. It can be observed that the participants with the ESRRβ CT allele show significantly ($p \leq 0.05$) poorer hearing threshold at 2, 3, 4 and 6 kHz in right ear and at 4 and 6 kHz in left ear before the noise exposure.

Table 4 shows tympanometric data for individuals with ESRRβ rs61742642 CC vs. CT genotype. There was no statistically significant ($p \leq 0.05$) group difference observed for tympanometric measures such as ear canal volume, compliance, middle ear pressure and gradient in both ears. This result suggests that the group differences in theimmittance measurements are small enough that they are clinically non-significant between the ESRRβ groups. Therefore, group differences in the immittance measurements are not likely to affect otoacoustic emissions between the ESRRβ groups.

Table 5 shows reflexometric findings for both the ESRRβ groups. It was observed that the acoustic reflex thresholds were not significantly different ($p \leq 0.05$) between participants with ESRRβ CC vs. CT genotypes for both ears. There was no significant

difference observed between the both ESRRβ groups for the reflexometric variables (Table 5).

Table 3. Descriptive Statistics of Pre-exposure Audiometric Thresholds for Participants with ESRRβ rs61742642 CC vs. CT Genotype

Hearing Threshold	ESRRβ	Mean	SD	t	p-value
HT Right ear – 1 kHz	CC	2.25	4.929	-.848	.400
	CT	3.42	5.015		
HT Right ear- 2 kHz	CC	-.13	4.598	-2.092	**.041**
	CT	2.37	3.483		
HT Right ear- 3 kHz	CC	3.50	4.414	-2.746	**.008**
	CT	6.05	2.677		
HT Right ear- 4 kHz	CC	2.63	5.309	-2.686	**.009**
	CT	6.84	6.283		
HT Right ear- 6 kHz	CC	1.63	4.584	-2.916	**.005**
	CT	5.53	5.243		
HT Right ear- 8 kHz	CC	3.63	7.844	-.543	.589
	CT	4.74	6.118		
HT Left ear-1 kHz	CC	-.50	4.777	-1.510	.137
	CT	1.58	5.284		
HT Left ear- 2 kHz	CC	-1.75	4.168	-1.978	.053
	CT	.53	4.047		
HT Left ear- 3 kHz	CC	2.38	3.753	-.723	.472
	CT	3.16	4.153		
HT Left ear- 4 kHz	CC	3.63	4.527	-2.207	**.031**
	CT	6.84	6.500		
HT Left ear- 6 kHz	CC	2.38	5.990	-2.281	**.026**
	CT	6.32	6.634		

HT Left ear- 8 kHz	CC	.75	4.743	-1.068	0.295
	CT	2.63	6.946		

Table 4. Descriptive Statistics of Tympanometric Variables for Participants with the ESRRβ rs61742642 CC vs. CT Genotype

	ESRRβ	Mean	SD	t	p-value
Ear canal volume in cm^3 (left ear)	C/C	1.16	.288	-.514	.610
	C/T	1.20	.297		
Compliance in cm^3 (left ear)	C/C	.67	.189	-.937	.353
	C/T	.72	.263		
Middle Ear Pressure in dapa (left ear)	C/C	-34.12	9.787	.790	.433
	C/T	-36.73	15.423		
Gradient in dapa (left ear)	C/C	76.82	15.980	.020	.984
	C/T	76.73	15.750		
Ear canal volume in cm^3 (right ear)	C/C	1.13	.245	-1.256	.220
	C/T	1.24	.348		
Compliance in cm^3 (right ear)	C/C	.67	.292	-.206	.837
	C/T	.69	.278		
Middle Ear Pressure in dapa (right ear)	C/C	-45.00	25.945	-.774	.442
	C/T	-40.00	15.545		
Gradient in data (right ear)	C/C	76.87	28.789	-1.159	.251
	C/T	85.84	25.447		

Table 5. Descriptive Statistics of Reflexometry for Participants with the ESRRβ rs61742642 CC vs. CT Genotype

Acoustic Reflex (AR) Threshold	ESRRβ	N	N (absent AR)	Mean (in dB)	SD	T	p-value
500 Hz (left ear)	C/C	38	1	95.92	4.62	-.264	.794
	C/T	18	1	96.39	6.81		
1 kHz (left ear)	C/C	39	0	96.54	5.39	.094	.925
	C/T	18	1	96.39	5.89		
2 kHz (left ear)	C/C	37	2	94.32	5.54	-.957	.343
	C/T	16	3	95.94	5.83		
500 Hz (right ear)	C/C	39	0	97.17	6.26	-.184	.855
	C/T	18	1	97.50	5.75		
1 kHz (right ear)	C/C	38	1	96.97	5.39	-.997	.323
	C/T	17	2	98.52	5.23		
2 kHz (right ear)	C/C	38	1	94.73	6.14	-.798	.428
	C/T	17	2	96.17	6.25		

Table 6 shows descriptive data for recent acoustic exposure, music exposure profile, noise exposure profile and eye color. It was found that 3 individuals (with ESRRβ CC allele) smoke tobacco on a regular basis in the entire sample (N = 59).

Table 7 shows the multiple regression analysis for pre-exposure DPOAE level. The ESRRβ polymorphism (β = -1.409 dB, CI = -2.662 – -0.156, p = 0.028) and music exposure (β = -0.0608 dB, CI = -1.175 – -0.04, p = 0.036) are statistically significant predictors of the pre-exposure DPOAE level after adjusting for the other variables in the model. The regression analysis suggests that participants with ESRRβ CT genotype

exhibit statistically significant reduction in pre-exposure DPOAE levels compared to participants with ESRRβ CC genotype.

Table 6. Descriptive Statistics for the Experimental Variables

	ESRRβ		Total
Recent Acoustic Exposure	CC	CT	
0-2 hours	3	1	4
2-12 hours	5	3	8
12-48 hours	14	3	17
> 48 hours	18	12	30
Total	40	19	59
Music Exposure	CC	CT	
1 (0-82)	9	5	14
2 (83-141)	12	3	15
3 (142-240)	10	5	15
4 (>240)	9	6	15
Total	40	19	59
Noise Exposure	CC	CT	
No Noise exposure	25	10	35
At least one incidence of routine noise exposure	12	9	21
More than one incidences of routine noise exposures	3	0	3
Total	40	19	59
Eye Color	CC	CT	
Blue	12	7	19
Green	4	2	6
Hazel	8	4	12
Brown	16	6	22
Total	40	19	59

Table 7. Multiple Linear Regression Analysis for Pre-exposure DPOAE Level

Coefficients							
Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
Regression Constant	11.318	2.849		3.972	.000	5.598	17.038
ESRRβ	-1.409	.624	-.288	-2.258	**.028**	-2.662	-.156
Smoking	2.704	1.428	.260	1.893	.064	-.164	5.571
Recent Acoustic Exposure	-.345	.332	-.140	-1.040	.303	-1.011	.321
Music exposure	-.608	.283	-.295	-2.150	**.036**	-1.175	-.040
Eye color	.228	.680	.048	.335	.739	-1.137	1.593
Noise Exposure	-.281	.498	-.073	-.563	.576	-1.281	.720
Gender	.140	.604	.030	.233	.817	-1.072	1.353

Dependent Variable: Pre-exposure Overall DPOAE (in dB SPL)

Adjusted R Square = 0.097

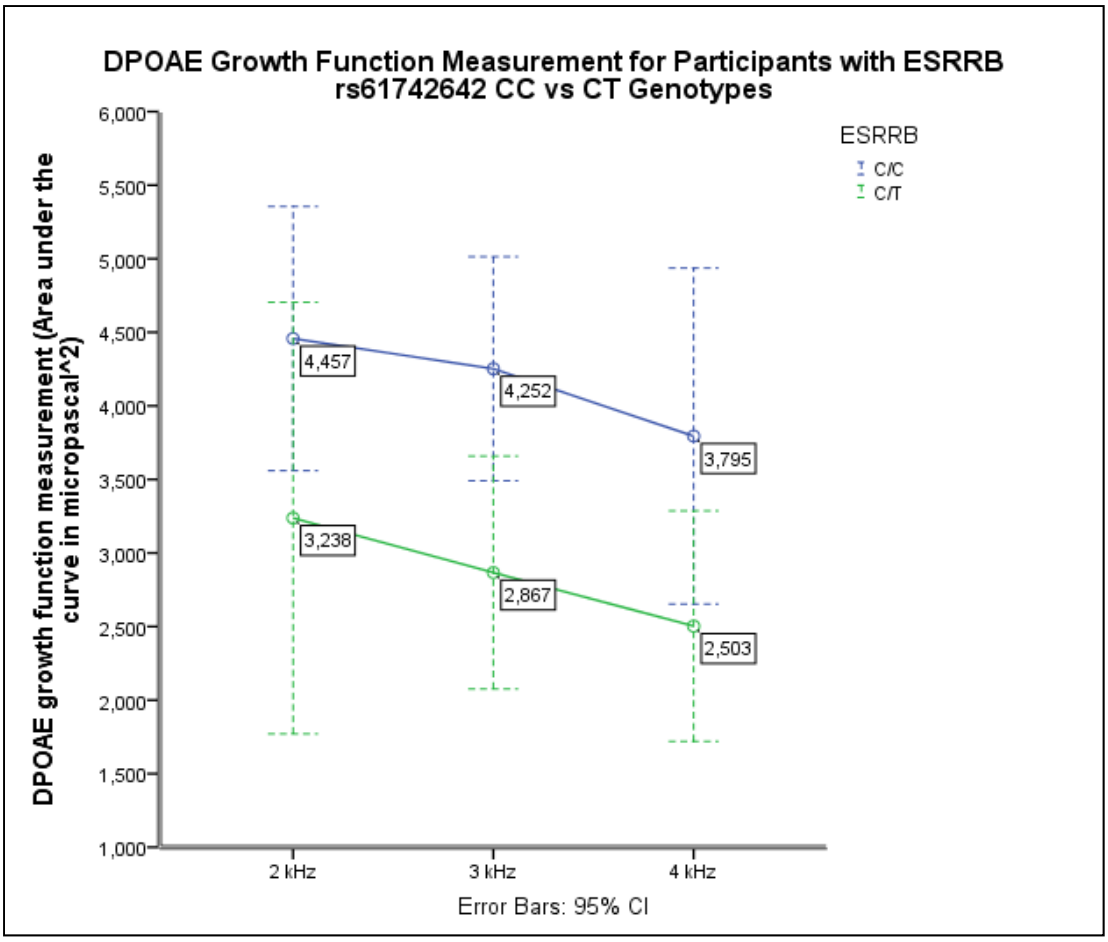

Figure 3. Pre-exposure DPOAE Amplitude between Individuals with ESRRβ CC and CT Genotype

Table 8. Multiple Linear Regression Analysis for Pre-exposure TEOAE Amplitude

Coefficients						
Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
Regression Constant	11.430	5.133		2.227	.031	1.115	21.746
ESRRβ	-1.088	1.131	-.128	-.962	.341	-3.360	1.184
Smoking	3.407	2.563	.190	1.330	.190	-1.743	8.558
Recent Acoustic Exposure	-1.039	.601	-.244	-1.729	.090	-2.247	.169
Music exposure	-.675	.514	-.187	-1.312	.196	-1.708	.359
Eye color	1.020	1.242	.123	.821	.416	-1.477	3.517
Noise Exposure	-.588	.903	-.088	-.651	.518	-2.403	1.227
Gender	-.865	1.094	-.107	-.790	.433	-3.063	1.333

Dependent Variable: Baseline overall TEOAE amplitude (in dB SPL)
Adjusted R Square = 0.033

Table 8 shows a multiple regression analysis of the TEOAE data. Two participants (1 male and 1 female from the CC group) were excluded from the TEOAE analysis as their TEOAE recordings were showing more than 20 rejected sweeps. A linear regression model revealed that the unstandardized coefficient for ESRRβ was -1.088 dB, which was not statistically significant ($p = 0.341$) after statistically controlling

for gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile.

TEOAE amplitude is not a frequency specific measurement, as it is evoked using click stimuli. Therefore, TEOAE data was further analyzed using a signal-to-noise ratio measurement across the frequency range from 1 to 4 kHz. It was expected that TEOAE measures would exhibit statistically significant group differences at frequency bands around 2 to 4 kHz because the baseline DPOAE amplitude difference was found to be statistically significant between the ESRRβ groups in the 2 to 4 kHz frequency range. TEOAE signal-to-noise ratios were calculated by subtracting TEOAE amplitude values (in dB SPL) from the noise floor (in dB SPL) for 5 frequency bands (1/3 octaves) from 1 to 4 kHz frequency range. Signal-to-noise ratio values were considered to be zero in the frequency band where amplitude of the signal was below the noise floor. A Repeated Measure ANOVA with 5 within subject factors (5 frequencies) and 2 between subject factors (ESRRβ and gender) was utilized to evaluate group differences. The main effect of ESRRβ ($F = 5.023$, $p = 0.026$, Figure 4) and gender ($F = 5.037$, $p = 0.029$, Figure 5) were statistically significant (Table 9). The data further indicate that the participants with the ESRRβ CT genotype show statistically significant reduction in TEOAE signal-to-noise ratios in the frequency range from 1 to 2 kHz (Figure 5).

Table 9. Summary of Repeated Measure ANOVA: Main Effect of ESRRβ, Gender and ESRRβ-Gender Interaction on TEOAE SNRs

Source	Mean Square	Df	F	Sig.
Intercept	61347.431	1	592.324	<.000001
ESRRβ	541.649	1	5.230	**.026**
Gender	521.718	1	5.037	**.029**
ESRRβ * Gender	57.697	1	.557	.459
Error	103.571	53		

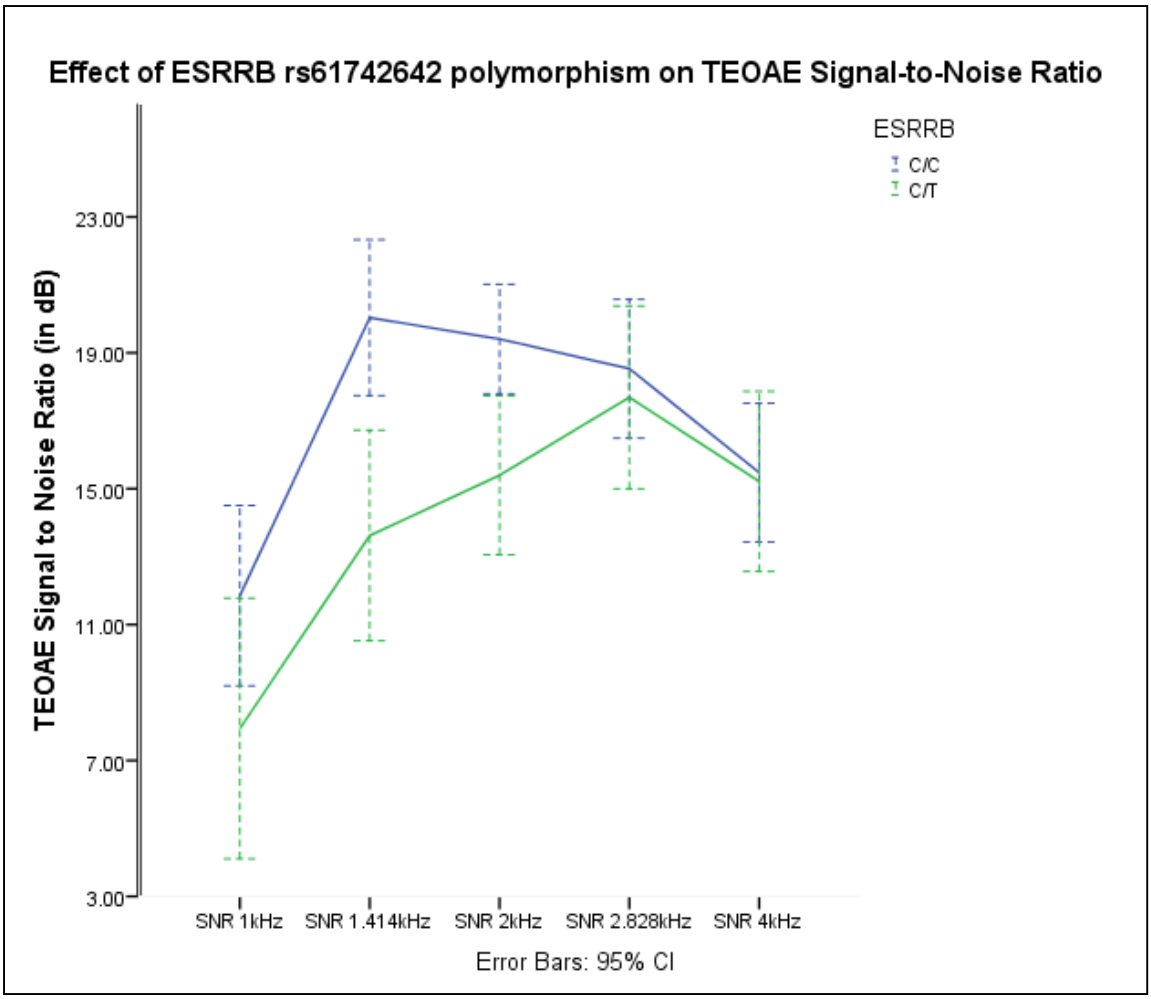

Figure 4. ESRRβ rs61742642 Polymorphism and Pre-exposure TEOAE Signal-to Noise Ratios

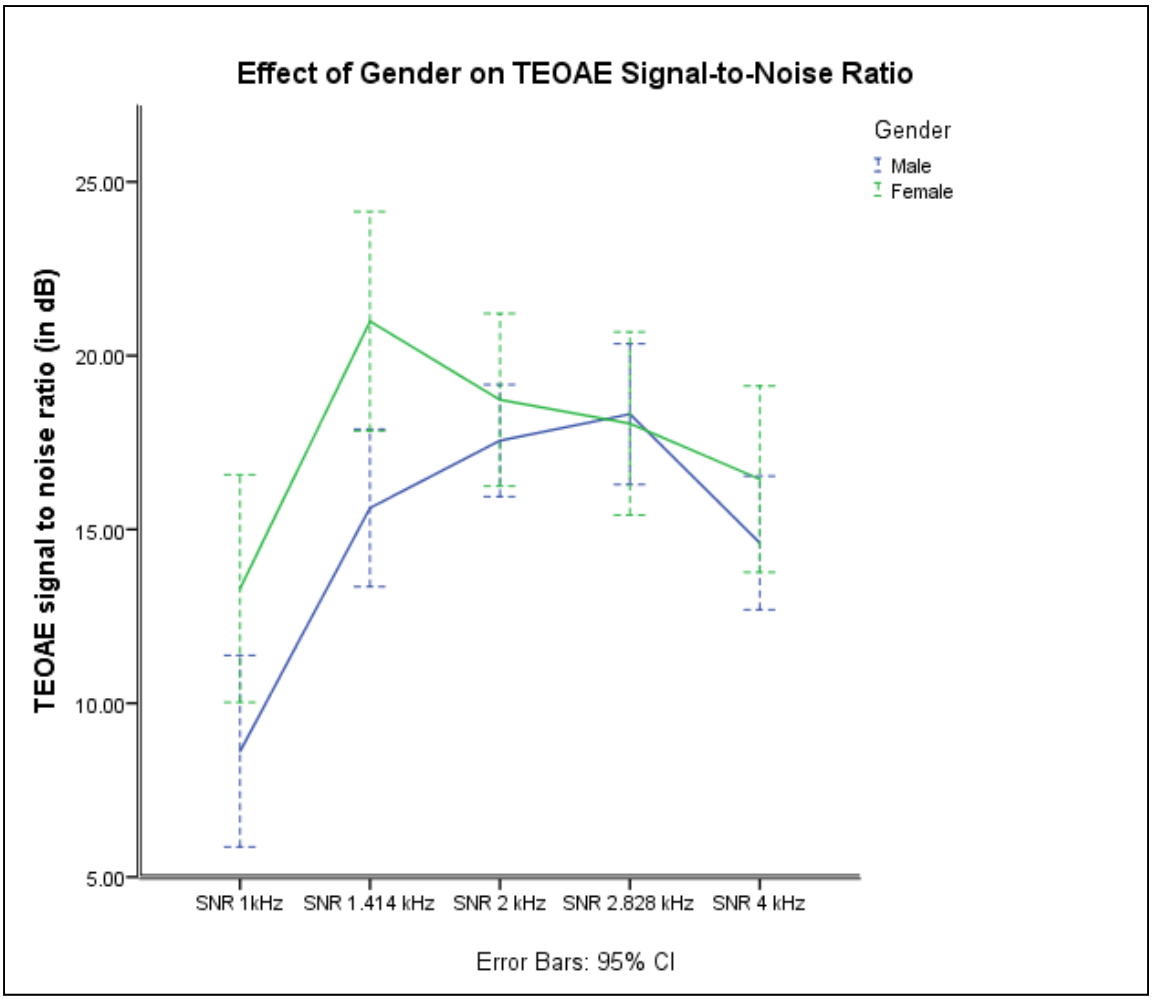

Figure 5. Gender and TEOAE Signal-to-Noise Ratios

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Table 10. A Multiple Linear Regression Analysis of Pre-exposure Contralateral Suppression of TEOAE

Coefficients							
Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
Regression Constant	-.109	.807		-.136	.893	-1.730	1.512
ESRRβ	-.424	.178	-.322	-2.387	**.021**	-.781	-.067
Smoking	.518	.403	.186	1.286	.204	-.291	1.327
Recent Acoustic Exposure	.116	.094	.174	1.224	.227	-.074	.305
Music exposure	.010	.081	.017	.119	.906	-.153	.172
Eye color	-.085	.195	-.066	-.436	.665	-.477	.307
Noise Exposure	-.003	.142	-.003	-.023	.981	-.288	.282
Gender	-.022	.172	-.018	-.129	.898	-.368	.323

Dependent Variable: TEOAE suppression (in dB SPL)
Adjusted R Square = 0.032

TEOAE suppression was calculated by subtracting the TEOAE amplitude recorded with 50 dB SL contralateral broadband noise from the TEOAE amplitude recorded without the contralateral noise. A multiple linear regression analysis was performed on contralateral suppression of TEOAE with variables previously associated with NIHL. ESRRβ polymorphism, smoking habits, recent acoustic exposure history, eye color, gender, noise exposure profile and music exposure profile were used in the analysis. Table 10 shows that participants with ESRRβ rs61742642 CT polymorphism exhibited a statistically significant reduction in the amount of TEOAE contralateral suppression (beta = - 0.424 dB, p = 0.021, Figure 6).

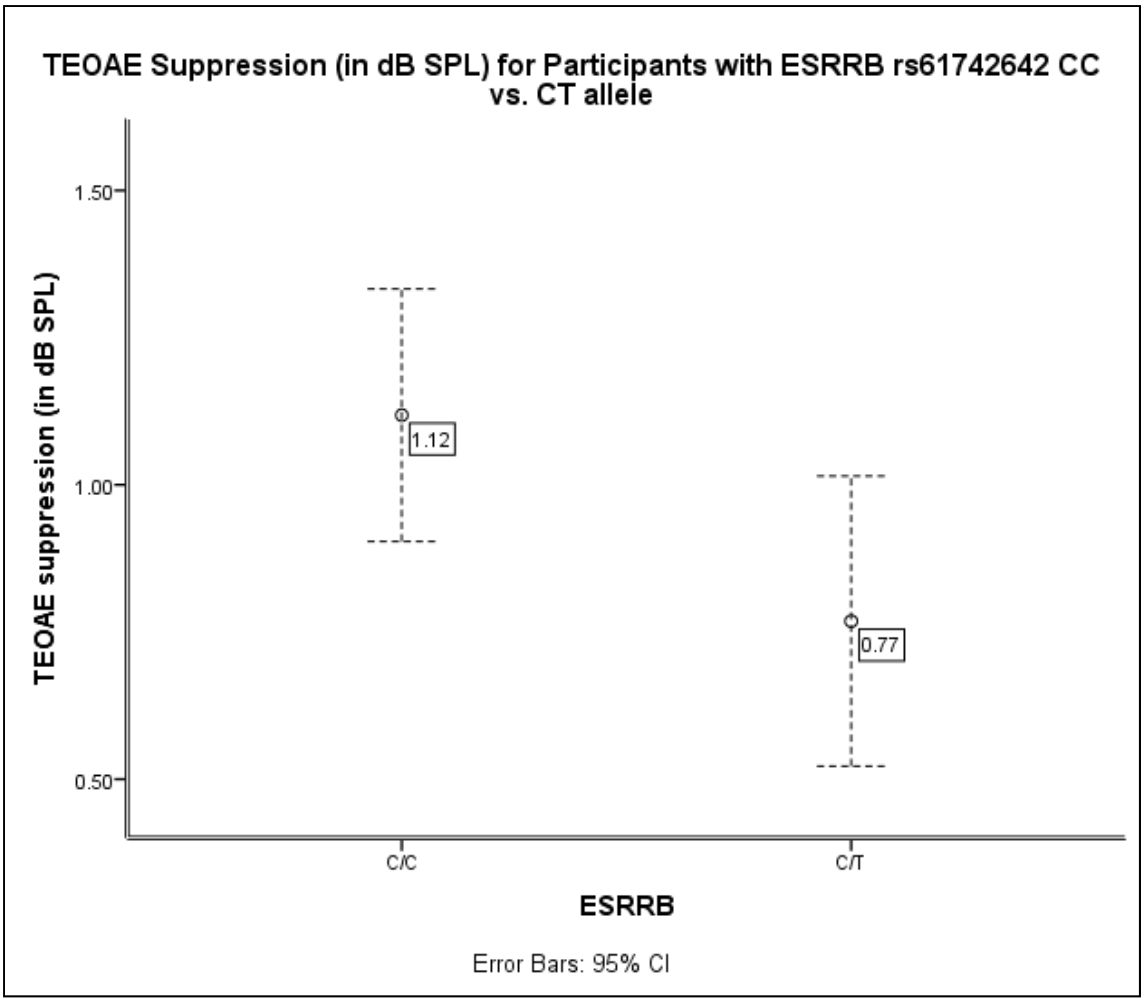

Figure 6. ESRRβ rs61742642 Polymorphism and Pre-exposure TEOAE Suppression

Results of the Hypotheses Testing

Hypothesis 1: Participants with the ESRRβ rs61742642 CT genotype will exhibit increased Audiometric Temporary Threshold Shift (ATTS) compared to participants with the ESRRβ CC genotype following the 10 minutes of 90 dB SL narrow-band noise exposure after statistically controlling for variables previously associated with temporary NIHL. ESRRβ polymorphism, gender, smoking, eye color, recent acoustic exposure history, noise exposure profile, music exposure profile and counterbalancing between audiometry and DPOAE was also included in the multiple linear regression analysis of the ATTS data.

Table 11 shows results of the multiple linear regression analysis. The ESRRβ polymorphism is a statistically significant predictor of ATTS (β= 10.498 dB, CI = 6.413 – 14.583, $t(51) = 5.162$, $p < 0.001$). The analysis indicates that the participants with the ESRRβ rs61742642 CT genotype acquire 10.498 dB higher ATTS compared to participants with ESRRβ rs61742642 CC genotype (beta = 10.498, CI = 6.413 – 14.583, $p < 0.0001$). The multiple regression analysis further indicates that the counterbalance effect is another statistically significant predictor of ATTS (β = -8.005, CI = -12.106 – -3.904, $p < 0.0003$). The result indicates that individuals tested with audiometry before DPOAE following the noise exposure show a statistically significant reduction in ATTS compared with individuals tested with DPOAE before audiometry.

Table 11. Regression Analysis: Predictors of Audiometric Temporary Threshold Shift

Coefficients							
Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
Regression Constant	20.359	9.320		2.184	.034	1.640	39.078
ESRRβ	**10.498**	2.034	.525	5.162	**<.0001**	6.413	14.583
Smoking	-3.195	4.725	-.075	-.676	.502	-12.685	6.296
Recent Acoustic Exposure	1.479	1.081	.147	1.368	.177	-.693	3.651
Music exposure	.017	.921	.002	.018	.986	-1.832	1.866
Eye color	4.279	2.337	.222	1.831	.073	-.414	8.973
Noise Exposure	1.601	1.624	.101	.986	.329	-1.661	4.862
Counterbalance	**-8.005**	2.042	-.428	-3.921	**<.0003**	-12.106	-3.904
Gender	2.413	1.976	.128	1.221	.228	-1.556	6.382

Dependent Variable: Audiometric Temporary Threshold Shift (in dB)
Adjusted R Square = 0.426

Table 12 shows a correlation matrix for the experimental variables. It was found that eye color was statistically significantly correlated with the counterbalance effect ($r = 0.364$, $p = 0.005$) and music-exposure profile ($r = 0.33$, $p = 0.011$). Counterbalance was assigned randomly to participants. It happened by chance that individuals with brown eyes were tested more frequently with DPOAE following noise exposure, and individuals with lighter eyes were tested more frequently with audiometry following noise exposure. It also happened by chance that individuals with higher music exposure had brown eyes, and individuals with lower music exposure had lighter eyes.

The multiple linear regression analysis found that the confidence interval of the unstandardized co-efficient beta is greater than 5 dB which supports the first hypothesis. It was concluded that individuals with the CT genotype acquire clinically higher ATTS compared with the individuals carrying the CC genotype.

Table 12. Cross-Correlation Matrix for the Experimental Variables

	ATTS	DPTLS	TETLS	ESRRβ	G	CB	Eye	Sm	RAEx	MEx	NEx
ATTS	1										
DPTLS	-.293*	1									
TETLS	-.080	.230	1								
ESRRβ	.523***	-.265*	-.077	1							
G	.203	-.077	.096	.004	1						
CB	-.401**	.203	-.111	-.025	-.049	1					
Eye	-.022	.070	.071	-.081	.165	.364**	1				
Sm	-.029	-.008	-.007	.160	-.042	.228	.178	1			
RAEx	.179	-.254	-.183	.098	.109	-.069	-.197	.226	1		
MEx	.062	-.247	-.073	.066	.190	.115	.330*	.249	.011	1	
NEx	.089	.066	-.132	.019	-.032	-.073	-.241	-.082	-.013	-.056	1

Note:

ATTS = Audiometric Temporary Threshold Shift
DPTLS = DPOAE Temporary Level Shift
TETLS = TEOAE Temporary Level Shift
ESRRβ = Estrogen-Related Receptor Beta
G = Gender
CB = Counterbalance effect
Eye = Eye color
Sm = Smoking Yes/No
RAEx = Recent Acoustic Exposure
MEx = Music Exposure Profile
NEx = Noise Exposure Profile

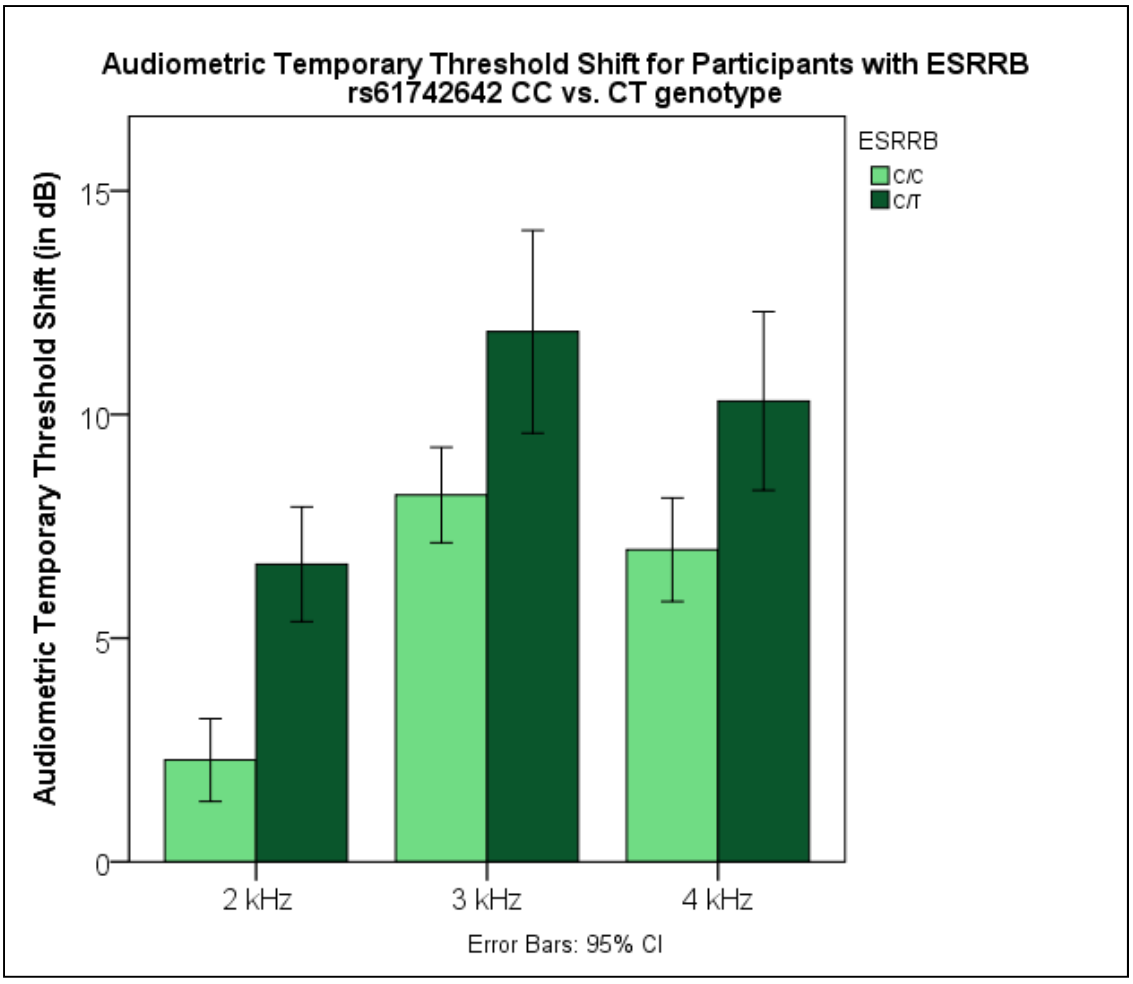

Figure 7. ATTS at 2, 3 and 4 kHz between Individuals with ESRRβ rs61742642 CC vs. CT Genotype

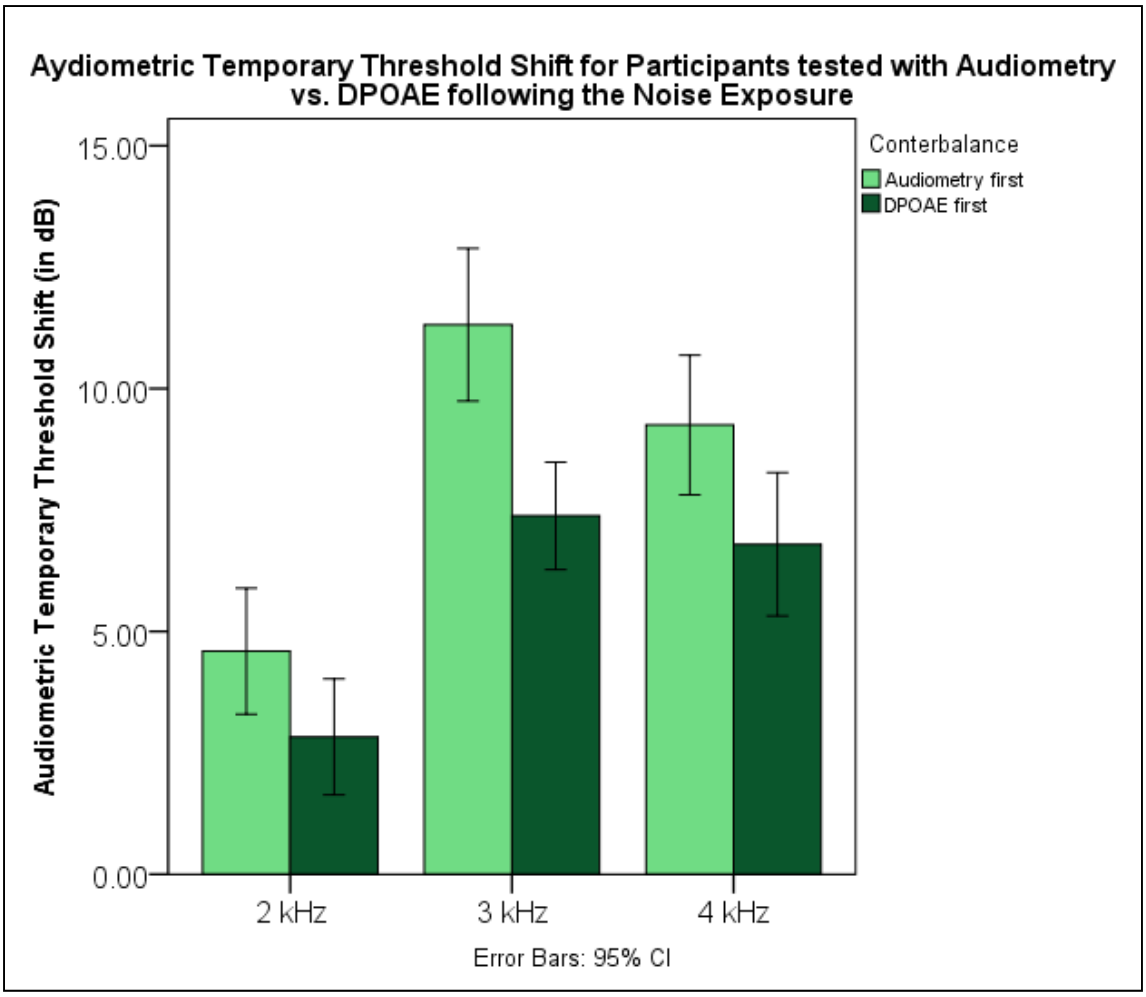

Figure 8. ATTS for Participants Tested with Audiometry vs. DPOAE Following the Experimental Noise Exposure

Hypothesis 2: There would be no clinical difference between overall Distortion Product otoacoustic emission Temporary Level Shift (DPTLS) between participants with ESRRβ rs61742642 CT genotype compared to participants with ESRRβ CC genotype after statistically controlling for variables previously associated with temporary NIHL like gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile. Counterbalancing between audiometry and DPOAE was also included in the multiple linear regression analysis of the DPTLS data.

Table 13 shows the results of the regression analysis on DPTLS data. The analysis suggests that individuals with ESRRβ CT genotype do not acquire significantly higher DPTLS compared to individuals with ESRRβ CC genotype as the upper and lower limits of the confidence interval for the regression coefficient was within the range of 0.87 and -0.87 dB (β = -0.037 dB, CI = -0.663 – 0.589).

Table 13. Regression Analysis: Predictors of DPOAE Temporary Level Shift

Coefficients							
Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
Regression Constant	.515	1.429		.361	.720	-2.354	3.385
ESRRβ	-.037	.312	-.016	-.118	.906	-.663	.589
Smoking	.404	.724	.082	.558	.580	-1.051	1.859
Recent Acoustic Exposure	-.259	.166	-.223	-1.565	.124	-.592	.073
Music exposure	-.152	.141	-.157	-1.080	.285	-.436	.131
Eye color	-.078	.358	-.035	-.219	.828	-.798	.641
Noise Exposure	.043	.249	.024	.173	.864	-.457	.543
Counterbalance	.539	.313	.250	1.722	.091	-.090	1.168
Gender	.164	.303	.075	.541	.591	-.444	.772

Dependent Variable: DPOAE Temporary Level Shift (in dB SPL)
Adjusted R Square = -0.012

Hypothesis 3: There would be no clinical difference between overall TEOAE level shift (in dB) between participants with ESRRβ rs61742642 CT genotype compared to participants with ESRRβ CC genotype after statistically controlling for variables previously associated with temporary NIHL like gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile. The counterbalance effect was not included in the analysis as TEOAE was collected after audiometry and DPOAE for all participants.

A multiple regression analysis was run on the TEOAE temporary level shift data (Table 14). The results indicate that the lower limit of the 95% confidence interval of the unstandardized ESRRβ coefficient is less than the threshold of clinical significance – 1.2 dB (β = -0.467 dB, CI = -1.573- 0.640, p = 0.401). The results indicate that there was insufficient evidence to support hypothesis 3. However, it was observed that the upper limit of the confidence interval was less than 1.2 dB (β = -0.467 dB, CI = -1.573- 0.640, p = 0.401) which suggests that individuals with the ESRRβ CT genotype did not acquire clinically higher TETLS compared to individuals with the ESRRβ CC genotype.

Table 14. Regression Analysis: Predictors of TEOAE Temporary Level Shift

Coefficients							
Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
Regression Constant	.147	2.501		.059	.953	-4.878	5.173
ESRRβ	-.467	.551	-.118	-.847	.401	-1.573	.640
Smoking	.632	1.248	.076	.506	.615	-1.877	3.141
Recent Acoustic Exposure	-.380	.293	-.191	-1.299	.200	-.969	.208
Music exposure	-.087	.251	-.052	-.347	.730	-.590	.417
Eye color	-.024	.605	-.006	-.039	.969	-1.240	1.193
Noise Exposure	-.591	.440	-.188	-1.343	.185	-1.475	.293
Gender	.485	.533	.128	.910	.367	-.586	1.556

Dependent Variable: TEOAE Temporary Level Shift (in dB SPL)
Adjusted R Square = -0.030

Hypothesis 4: Participants with the ESRRβ rs61742642 CT genotype will exhibit significantly reduced TEOAE temporary suppression shift (in dB) compared to participants with the ESRRβ CC genotype after statistically controlling for variables previously associated with temporary NIHL like gender, smoking, eye color, recent acoustic exposure history, noise exposure profile and music exposure profile.

The hypothesis was tested by running a multiple linear regression analysis (Table 15). The regression coefficient for ESRRβ is 0.224 dB which is not statistically significant (CI = -0.111 - 0.559, $p = 0.186$). The results provide insufficient statistical evidence to conclude that individuals with the ESRRβ CT genotype exhibit a significantly reduced TEOAE suppression shift compared to individuals with the ESRRβ CC genotype as the upper limit of the confidence interval is greater than the previously defined clinical significance threshold (i.e. 0.10 dB) with $p > 0.05$.

Table 15. Regression Analysis: Predictors of TEOAE Temporary Suppression Shift

Coefficients							
Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
Regression Constant	-.678	.760		-.893	.376	-2.206	.849
ESRRβ	.224	.167	.177	1.343	.186	-.111	.559
Smoking	.662	.384	.248	1.725	.091	-.110	1.434
Recent Acoustic Exposure	-.113	.089	-.178	-1.275	.209	-.291	.065
Music exposure	-.120	.076	-.223	-1.576	.122	-.272	.033
Eye color	-.223	.194	-.181	-1.147	.257	-.614	.168
Noise Exposure	-.174	.133	-.174	-1.305	.198	-.442	.094
Counterbalance	.012	.169	.010	.071	.944	-.328	.352
Gender	.320	.162	.265	1.978	.054	-.005	.645

Dependent Variable: TEOAE Temporary Suppression Shift (in dB SPL)
Adjusted R Square = 0.071

CHAPTER V

DISCUSSION

The major finding of the present study is that musicians with ESRRβ rs61742642 CT genotype showed significantly higher audiometric temporary threshold shifts (ATTS) without significantly different DPOAE temporary level shifts (DPTLS) compared to musicians with ESRRβ rs61742642 CC genotype. All participants acquired a temporary decrement in audiometric thresholds and OAE amplitude following the noise exposure. This result is in agreement with previous studies on temporary NIHL suggesting that the noise exposure used in the current study is effective to induce temporary changes in auditory physiology which can be measured by audiometry and OAE test battery (Marshall & Heller, 1998 and Attias, Sapir, Bresloff, Reshef-Haran, & Ising, 2004).
A multiple linear regression analysis of TETLS data showed that the unstandardized ESRRβ regression coefficient is -0.467 dB (CI = -1.573- 0.640, p = 0.401) with the lower confidence interval -1.573 which is lower than the threshold of clinical significance (i.e.-1.2 dB). The results provided insufficient statistical support for the hypothesis 3 because it could not eliminate the possibility of individuals with ESRRβ CT genotype acquiring lower TETLS compared to individuals with the ESRRβ CC genotype. This observation can be attributed to measurement of overall TEOAE amplitude. Click evoked TEOAEs are comprised of multiple frequencies and frequency specific TEOAE amplitude is more sensitive to identify lesion specific cochlear damage compared to overall TEOAE

amplitude (Marshall & Heller, 1998). Jedrzejczak, Blinowska, & Konopka (2005) suggested that time-frequency analysis of TEOAE responses can be sensitive to identify changes in the spectral aspects of TEOAE responses. A post-hoc time-frequency analysis of TEOAEs can be utilized to further test the hypothesis 3. However, the present results suggest that individuals with the ESRRβ CT genotype did not acquire clinically higher TETLS compared to individuals with the ESRRβ CT genotype as the upper limit of the confidence interval is lower than the threshold of clinical significance (i.e. 1.2 dB). Individuals with the ESRRβ CT genotype acquired a mean of 10.5 dB higher ATTS without acquiring significantly higher DPTLS and TETLS compared with their counterparts. The finding suggests that these increments in ATTS cannot be attributed to the mechanical properties of the cochlea because DPTLS and TETLS are not significantly higher for individuals with ESRRβ CT genotype compared with individuals carrying the ESRRβ CC genotype. Previous investigators have suggested that changes in audiometric threshold without a concomitant change in OAEs are a sign of metabolic compromise in the cochlea which can be a result of a decrement in the endolymphatic potential (Mills, Norton, & Rubel, 1993 and Gates et al., 2002). The metabolic distress can reduce the gain of the cochlear amplifier (i.e. hearing sensitivity measured by audiometry) without compromising its mechanical properties (i.e. waveform distortion measured by OAEs). The present study indicates that individuals with the ESRRβ CT genotype show signs of metabolic distress following the noise exposure.

Potential Physiological Mechanisms Underlying ESRRβ Polymorphism-related Susceptibility to NIHL

Effects of ESRRβ Polymorphism on Potassium Ion Circulation in the Cochlea

Multiple physiological mechanisms may explain predominant metabolic compromise in the cochlear physiology following noise exposure in individuals with the ESRRβ CT genotype compared with their counterparts. ESRRβ, an evolutionarily highly conserved protein, is expressed in important cochlear structures like the stria vascularis, spiral ligament, Reissner's membrane, supporting cells of the outer and inner hair cells, spiral ganglion and the auditory nerve, all of which are critical to maintain hearing sensitivity (Collin et al., 2008). ESRRβ is a nuclear receptor which regulates the transcription of other cochlear genes associated with fluid and redox homeostasis of cochlear structures (Raghuram et al., 2007). Mutations in ESRRβ causes profound hearing loss (Collin et al., 2008) which can be associated with its function in stria vascularis to regulate KCNE1, KCNQ1 and ATP1B2 which are essential to regulate endocochlear potential (Chen and Nathans, 2007). This suggests that ESRRβ is important to maintain overall cochlear physiology. ESRRβ protein molecules created by the ESRRβ gene with rs61742642 CT genotype replaces proline with serine in the ESRRβ amino acid sequence at P386S which builds a ligand binding pocket of the receptor. It is possible that the proline to serine replacement in people with the ESRRβ CT genotype can cause changes in the shape and/or chemistry of the ligand-binding domain of the ESRRβ protein. This may compromise ability of ESRRβ ligand to attach with the receptor and cause an inefficient ESRRβ protein molecule. The inefficient ESRRβ

protein may not respond efficiently to the redox state of the cochlear cell, and further may compromise its ability to regulate other genes. These include potassium ion regulator genes like KNCE, KCNQ1 and ATP1B2 (Chen and Nathans, 2007). Estrogen related-receptors are also important to mediate the effect of estrogens, thyroid hormone and glucocorticoid hormones important for cochlear development and homeostasis (Collin et al., 2008). Therefore, an inefficient ESRRβ protein molecule may interact poorly with regulated genes, hormones and other proteins maintaining the cascade of the nuclear receptor activated events. These inefficiencies may lead to increased susceptibility to NIHL.

This indication of metabolic distress observed in individuals with the ESRRβ CT genotype following noise exposure may be explained if ESRRβ is involved in the regulation of potassium ion movement in the cochlea. Maintenance of the endolymphatic potential is a major metabolic function of the stria vascularis. Regulated movement of the potassium ions into the endolymph is essential to maintain endolymphatic potential. Potassium ions are delivered directly from the blood to the epithelial cells of stria vascularis and they are pushed into the endolymph by a strategic array of basal, intermediate and marginal cells of the stria vascularis. Potassium ions are restored back to the stria vascularis by systematic refinement of K+ ion by the Reissner's membrane, supporting cells of the hair cells, tectorial membrane and external sulcus cells. ESRRβ is expressed in all of these cochlear structures. KCNE1 and KCNQ1 are transcriptional targets of ESRRβ and they are expresses in the marginal cells of the stria vascularis. KCNE1 and KCNQ1 regulated K+ ion channels are essential to transfer potassium ions

from the intrastrial fluid to the endolymph. ESRRβ regulates transcription of ATP1B2 (one subunit of the Na/K ATPase) and ATP1B1 (other subunit of the Na/K ATPase) in the stria vascularis and it is important for maintaining the sodium-potassium electrochemical gradient across the basal cells of the stria vascularis (Chen and Nathans, 2007). An inefficient ESRRβ might compromise regulation of the potassium ions circulation inside the cochlea to produce signs of metabolic distress observed in individuals with the ESRRβ CT genotype.

There might be multiple underlying mechanisms responsible for reducing potassium ion concentration in endolymph and subsequently endolymphatic potential following noise exposure which might be influenced by ESRRβ polymorphism. An inefficient ESRRβ protein might require a higher concentration of ligand molecules in the environment to get activated. This might lead to an increased threshold of ESRRβ sensitivity to respond to the redox cellular state. Therefore, the inefficient ESRRβ protein would take a longer time to respond to the redox cellular state leading to delayed regulation of KCNE1, KCNQ1, ATP1B2 and ATP1B1. This might significantly reduce potassium ion concentration inside the endolymph and causes sudden decrement in endolymphatic potential. Other possible mechanism to explain metabolic distress observed in individuals with the ESRRβ CT genotype is ligand-dependent activation of the ESRRβ receptor. The rs61742642 polymorphism replaces proline with serine in the ESRRβ amino acid sequence at P386S which builds a ligand binding pocket of the receptor which might compromise ability of the receptor to bind with its ligand. An inefficient ESRRβ protein may poorly interact with its ligand and physiologically

undershoot the ligand-dependent activity. Both mechanisms might cause poorer regulation of KCNE1, KCNQ1, ATP1B2 and ATP1B1 genes in the stria vascularis compared to a well-functioning ESRRβ protein. This could cause a decrement in endolymphatic potassium ion concentration as the K+ ion channels in the basal and marginal cells of the stria vascularis would not be regulated properly to meet with the physiological demands caused by noise exposure. This would lead to a sudden decrement in endolymphatic potential with minimum or no effect on the mechanical properties of the motion mechanism of the cochlea. Polymorphisms in KNCE1 and KCNQ1 were associated with NIHL in previous studies (Van Laer et al., 2006 and Konings et al., 2009a). Therefore, it is likely that an inefficient ESRRβ would have inefficiencies in modifying the ligand-dependent regulation of KCNE1 and KCNQ1, leading to increased susceptibility to NIHL.

Effects of ESRRβ Polymorphism on Management of Oxidative Stress

Another potential mechanism to explain signs of metabolic distress observed in individuals with the ESRRβ CT genotype is transfer of potassium ions through Reissner's membrane and supporting cells to the stria vascularis. Little is known about the role of ESRRβ in the Reissner's membrane and supporting cells of the cochlear hair cells. Reissner's membrane and supporting cells regulate potassium ion circulation inside the cochlea. Reissner's membrane and supporting cells show high metabolic activity and increased oxidative stress during noise exposure (Poirrier, Pincemail, Van Den Ackerveken, Lefebvre, & Malgrange, 2010). An inefficient ESRRβ protein may poorly respond to the redox cellular state of Reissner's membrane and supporting cells. This

would compromise regulation of potassium ion circulation genes that conduct potassium ions to the stria vascularis. Supporting cells have recently been found to produce a family of heat shock proteins to protect hair cells from the toxic molecules (May et al., 2013). ESRRβ may affect the expression of heat shock protein through its interactions with estrogen receptors to regulate redox cellular state (Aranda & Pascual, 2001 and Kumar, Saradhi, Chaturvedi, & Tyagi, 2006). An inefficient ESRRβ protein might be inefficient in its response to the oxidative stress induced by noise exposure in hair cells and their supporting cells. Oxidative stress can modulate the functioning of potassium ion channels (Liu & Gutterman, 2002). Therefore, the presence of the reactive oxygen species might slow down the conductance of potassium ions from the hair cells to the supporting cells and to the spiral ligament. This would result in a decrement of potassium ion influx to the stria vascularis which would further lead to a decrement in potassium ion concentration in endolymph and subsequent decrement in the endolymphatic potential.

Explanations of Unexpected Pre-exposure Audiometric and OAE Difference between the ESRRβ Groups

Individuals with the ESRRβ CT Genotype might Exhibit Longer Recovery from Temporary NIHL

An inefficient ESRRβ protein might take a longer period of time to produce the cascade of antioxidants-related rescue events in the cellular environment. Individuals with an inefficient ESRRβ protein might take a longer period of time compared with their counterparts to recover from temporary NIHL for the following reasons: (1) the need to restore more physiological functions as they acquire more metabolic compromise

following a brief acoustic exposure, and (2) an inefficient ESRRβ protein might not function well enough to restore the cochlear homeostasis. The present study evaluated effect of ESRRβ polymorphism on musicians who are exposed to loud music exceeding NIOSH noise exposure permissible standards on a regular basis (Phillips & Mace, 2008). Therefore, individuals with the inefficient ESRRβ protein might be exposed to loud music again before they recover completely from the previous exposure. This might explain the unexpected pre-exposure audiometric and OAE difference observed between the ESRRβ groups.

Impact of Longer Recovery Period on the Pre-exposure Audiometric and OAE Measurements

Individuals with the ESRRβ CT genotype show significantly poorer hearing thresholds in both ears pre-exposure, with significantly reduced DPOAE amplitude and TEOAE signal-to-noise ratio compared with their counterparts. There is no indication in the analysis of survey data suggesting that individuals with the ESRRβ CT genotype show significantly different variables previously associated with reduced OAE amplitude and audiometric thresholds such as recent acoustic exposure history, noise exposure profile, music exposure profile, gender, smoking habit and eye color. These pre-exposure results suggest that individuals with the ESRRβ CT genotype exhibit compromised outer hair cell physiology with a decrement in hearing sensitivity compared with their counterparts. This finding suggests that an inefficient ESRRβ protein may produce damage in the outer hair cell physiology even though ESRRβ is not expressed in the cochlear hair cells. Unpublished data from Phillips et al., (2012) suggests that the

audiometric hearing loss observed in the population of student individuals is likely to be temporary threshold shift as almost 80% of individuals do not show bilateral audiometric notch in subsequent audiometric testing. The DPTLS and TETLS findings suggest that individuals with the ESRRβ CT genotype acquire a compromise in outer hair cells physiology following noise exposure, but it is not significantly poorer than their counterparts. Previous research suggested that cochlear physiology recovers rapidly following noise exposure (Marshall & Heller, 1998 and Attias, Sapir, Bresloff, Reshef-Haran, & Ising, 2004). Therefore, the outer hair cell physiology is likely to recovery following the noise exposure for the both ESRRβ. It is possible that individuals with an inefficient ESRRβ protein might have slower outer hair cell recovery following noise exposure compared to their counterparts. This might be explained if the individuals with the inefficient ESRRβ protein did not recover completely from any exposure in the day prior to the testing session.

*Potential Mechanisms Explaining how Inefficient ESRR*β *Protein can lead to Outer Hair Cell Damage*

Little is known about the functioning of ESRRβ in the cochlea. Molecular pathways of ESRRβ activation and cascade of physiological events following its ligand-dependent activation are largely unknown. Recently ESRRβ was identified as a major nuclear receptor responsible for regulating the whole-body energy cycle (regulation of hunger, satiation and blood sugar levels) in a mouse-model (Byerly, Swanson, Wong, & Blackshaw, 2013). ESRRβ interacts with estrogen-related receptor γ and regulates functioning of the neural physiology in the hindbrain which is responsible for

maintaining the energy cycle. The cascade of ESRRβ interaction with estrogen-related receptors and their response genes may alter insulin sensitivity and blood glucose levels (Byerly, Swanson, Wong, & Blackshaw, 2013). Reduction in the blood glucose level has been associated with increased susceptibility to NIHL (Jang, Kim, Kwon, & Im, 2011). An inefficient ESRRβ protein might cause a slow-down in the maintenance of blood glucose levels necessary to provide energy to the hair cells and to their supporting cells to manage oxidative stress following noise exposure. This, in turn, might significantly increase risk of acquiring NIHL (Jang, Kim, Kwon, & Im, 2011). This can explain reduction of OAE amplitude and signal-to-noise ratio in individuals with the ESRRβ CT genotype compared to their counterparts.

Other possible mechanism related to OAE compromise and outer hair cell damage is a regulation of complex cascade events by ESRRβ. ESRRβ might interact with a complex network of thyroid receptors, estrogen-receptors, glucocorticoid receptors and produce a cascade of rescue events (Vanacker, Pettersson, Gustafsson, & Laudet, 1999 and Collin et al., 2008). ESRRβ shares a portion of its response element with estrogen receptor alpha (Vanacker, Pettersson, Gustafsson, & Laudet, 1999). Estrogen receptors work with a brain derived neurotrophic factor to protect hair cells, the spiral ganglion and the auditory nerve from the direct effect of noise exposure (Meltser et al., 2008). Deficiency of estrogen leads to significantly early onset of mid to high frequency age-related hearing loss which are related with hair cell dysfunction. An inefficient ESRRβ protein might poorly regulate estrogen receptors which subsequently would result in poor expression of estrogen-receptor beta and brain-derived neurotrophic factor to noise

induced reactive oxygen species. Poor management of reactive oxygen species can significantly increase the recovery period of temporary NIHL. Thyroid receptors are essential for developing and maintaining endolymphatic potential and it has been shown that ESRRβ can influence the thyroid hormone pathway by regulating receptor-interacting protein 140 (Castet et al., 2006). Thyroid receptors has been shown to modulate potassium conductance of the hair cells in the mouse cochlea (Rüsch, Erway, Oliver, Vennström, & Forrest, 1998). Therefore, an inefficient ESRRβ might influence the activity of the thyroid receptors which can subsequently lead to modulation of the potassium ion conductance, with possible negative consequences for the physiological recovery of the cochlear hair cells and susceptibility to NIHL (Van Laer et al., 2006). If this is the case, it may also explain why individuals with ESRRβ CT genotype exhibit poorer pre-exposure audiometric thresholds and reduced OAE amplitude and signal-to-noise ratio.

The present study found that individuals with the ESRRβ rs61742642 CT genotype exhibit significantly lower pre-exposure TEOAE suppression compared with their counterparts. ESRRβ is expressed abundantly in the spiral ganglion and auditory nerve cells. The function of ESRRβ in the central nervous system is not yet known, but estrogen-related receptors (α, β and γ) show a complex pattern of expression in the central neural network which might regulate gene expressions responsible for neural growth and maturation (Ren, Jiang, Ma, Nakaso, & Feng, 2011). An inefficient ESRRβ protein might not interact adequately with genes responsible for development and/or maintenance of central nervous system. This may lead to a compromise in the ability of

the nerve fibers to suppress and/or enhance incoming neural impulses. This inefficiency may lead to a decrement in the strength of the medial olivo-cochlear fibers to suppress cochlear responses (measured by the contralateral suppression of TEOAE). A decrement in the strength of TEOAE suppression is proposed as a physiological marker to NIHL susceptibility (Maison & Liberman, 2000). The medial olivo-cochlear nucleus innervates the outer hair cell and modifies cochlear responses when activated by contralateral acoustic stimuli. Reduction in the strength of the medial olivo-cochlear reflex leads to excessive vibration of the cochlear structures which can put an individual at risk for NIHL (Patuzzi & Thompson, 1991). Individuals with the ESRRβ CT genotype exhibit reduced suppression, suggesting reduced strength of the medial olivo-cochlear fibers to suppress cochlear activity, and therefore may have an increased susceptibility to NIHL. Therefore, reduced TEOAE suppression in individuals with ESRRβ CT genotype suggests that TEOAE contralateral suppression can be a potential endophenotype of NIHL susceptibility. Time-frequency analysis of TEOAE data should be used in future to further explore reduced strength of medial olivo-cochlear nerve fibers in individuals with ESRRβ CT genotype.

Implications of the Study

The present study utilized a battery of clinical audiologic tests with audiometry and otoacoustic emissions to explore the clinical effects of the previously associated ESRRβ rs61742642 polymorphism. Results of the present study suggest that individuals with ESRRβ rs61742642 CT genotype are susceptible to temporary NIHL. The underlying mechanism of ESRRβ rs61742642 CT genotype induced susceptibility to

NIHL is likely to be caused by metabolic distress in the stria vascularis or other cochlear structures. This study provides clinical evidence that genetic variability in the ESRRβ nuclear receptor is associated with individual susceptibility to NIHL. Almost 26% of participants with a bilateral notch were carriers of ESRRβ rs61742642 CT genotype as reported by Phillips et al., (2012). These results may be helpful in the development of genetic profiling to identify individuals at risk of acquiring NIHL.

The second implication of the present study is that clinical non-invasive testing can be utilized to explore the physiological basis of the NIHL susceptibility. Temporary NIHL induced by a controlled laboratory noise was utilized to evaluate changes in the cochlear physiology in individuals with the ESRRβ CC vs. CT genotype. A test battery was helpful to differentiate metabolic damage from the mechanical damage to the cochlear mechanism in individuals with the ESRRβ CT genotype. This study supports the concept that the underlying mechanisms leading to increased susceptibility to NIHL may be categorized as predominantly mechanical, metabolic and/or neural. A clinical test battery can be useful to identify mechanical, metabolic and/or neural basis of the NIHL susceptibility. This strategy can be helpful to overcome the replication failure often encountered in gene-environment association studies of NIHL as the test battery can provide more detail about the auditory physiology compared to a single test. This approach can provide insights into the underlying physiological mechanism by which an associated genotype leads to increased susceptibility to NIHL. This method can be utilized to define differential endophenotypes to explore the genetic basis of age-related

hearing loss and ototoxicity. This approach also can be useful to evaluate treatment efficacy of a pharmacological intervention in humans targeted to a specific gene.

Limitations of the Study

There are several limitations of this study. This is a volunteer sample study which limits the generalizability of the results as the sample may not be representative of the target population of college-age musicians. The statistical results should be treated with caution as the reason for the observed associations may not be due to the ESRRβ genotype but due to other genetic and/or environmental factors not studied in the present study. This study did not evaluate participants with the ESRRβ rs61742642 TT genotype as it is very rare. A larger study could also address this shortcoming. The study did not utilize a clinical TEOAE protocol to evaluate TEOAE suppression which compromised sensitivity of the OAE testing to appropriately evaluate changes in efferent suppression following noise exposure.

TEOAE contralateral suppression is measured usually with 60 dB SPL linear clicks with 30 to 50 dB SL contralateral broadband noise which produces significantly high TEOAE suppression (Collet, 1993). This study evaluated TEOAE suppression with 84 dB peSPL non-linear sweeps with 12.5 msec response window which was used to save post-exposure testing time. This method might compromise the sensitivity of the TEOAE suppression testing to evaluate changes in the strength of the medial olivo-cochlear bundle which might explain the failure to produce statistical support for Hypothesis 4. Further research is required to evaluate suppression of the otoacoustic emission using Collet's protocol for individuals with the ESRRβ CC vs. CT genotype to

explore the effects of ESRRβ rs61742642 polymorphism on efferent feedback following noise exposure.

Future Research Directions

It is still unclear whether the ESRRβ rs61742642 polymorphism is associated with permanent NIHL or not. A longitudinal study needs to be done with a battery of tests including OAEs and audiometry to explore if individuals with ESRRβ rs61742642 CT genotype acquire significantly higher and/or faster permanent hearing loss following repeated acoustic exposures or not compared with their CC counterparts.

TEOAE data collected in the current projects can be evaluated using advanced signal processing strategies to explore effects of ESRRβ rs61742642 polymorphism on outer hair cell physiology and efferent suppression. OAE data can be used to evaluate recovery function of the cochlear physiology following the noise exposure. Currently this is an ongoing project.

The physiological effects of ESRRβ rs61742642 polymorphism need to be studied in an animal model to explore the molecular basis of the NIHL susceptibility. The animal model can be utilized to check the effect of ESRRβ rs61742642 polymorphism on permanent NIHL, age-related hearing loss and ototoxicity.

A test battery with audiometry, otoacoustic emissions and electrocochleography needs to be used in future to identify different cochlear endophenotypes related with mechanical, metabolic and/or neural damage to the cochlear structures. The endophenotypes can be utilized to explore genetic susceptibility to NIHL, age-related hearing loss and ototoxicity. The genetic links to pure cochlear hearing loss can be useful

for risk profiling, individualized prevention planning and estimating treatment efficacy of the pharmaceutical interventions.

Conclusion

The present study evaluated the effects of a genetic polymorphism in human ESRRβ on cochlear physiology using a test battery of audiometry and otoacoustic emissions in young college-age individuals with relatively good health and hearing acuity. Individuals with the ESRRβ CT genotype acquired higher audiometric threshold shifts without significantly different DPOAE and TEOAE level shifts, suggesting that the nature of cochlear compromise in individuals with the ESRRβ CT genotype is metabolic (i.e. related with stria vascularis). Individuals with the ESRRβ CT genotype also exhibited significantly poorer pre-exposure audiometric thresholds and OAE amplitude. These results suggest that individuals with the ESRRβ CT genotype exhibit physiological indications of increased NIHL susceptibility. It is concluded that the audiologic test battery with audiometry and otoacoustic emissions is useful to differentially identify genetically-based metabolic vs. mechanical mechanisms underlying NIHL susceptibility.

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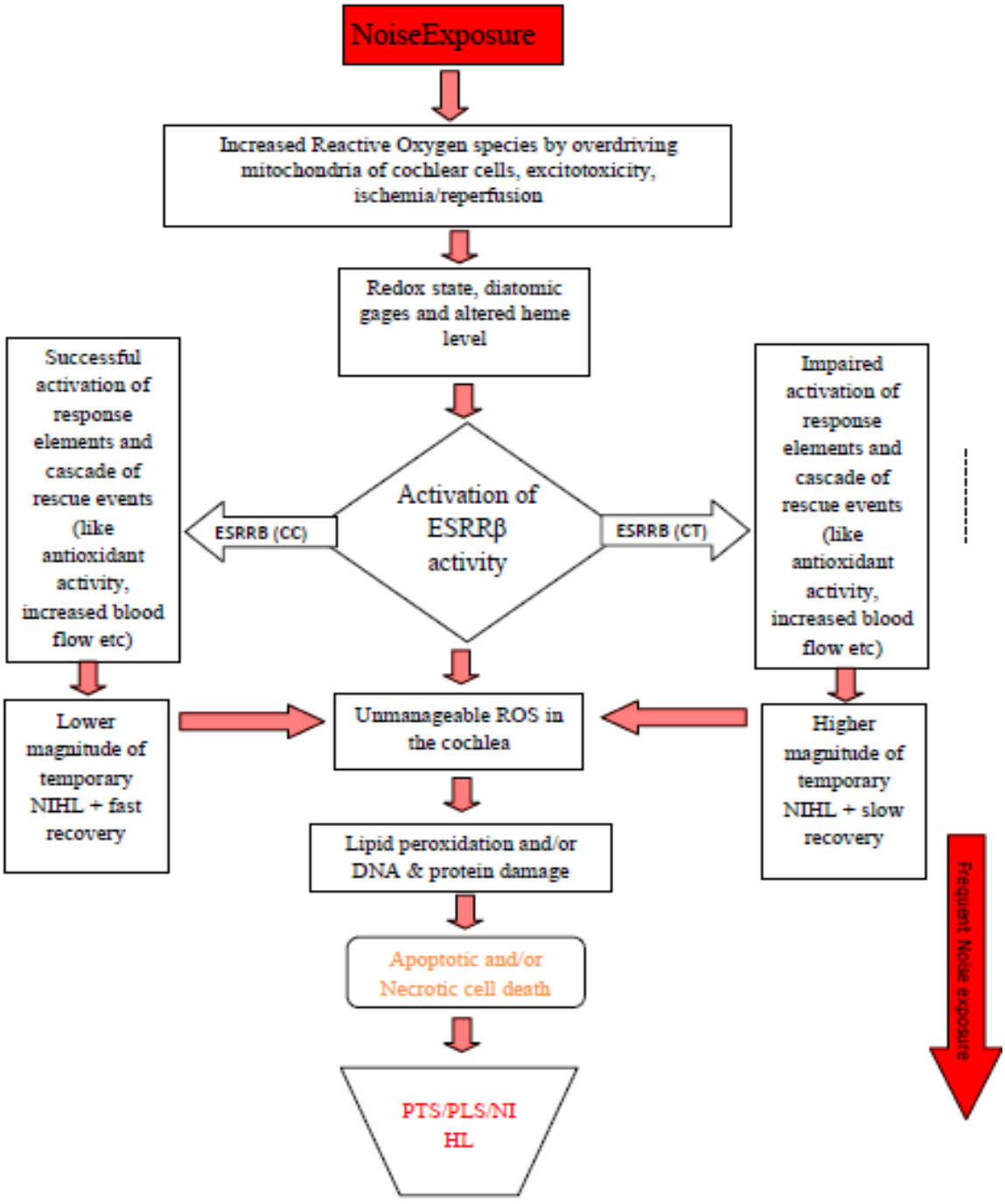
APPENDIX A

A THEORITICAL MODEL EXPLAINING ESRRβ rs61742642 SNP-RELATED RISK TO NIHL



APPENDIX B

SURVEY

Q1 I am 18 or older, and agree to participate in this research.

- ❍ Yes (1)
- ❍ No (2)

If No Is Selected, Then Skip To End of Survey

Q2 What is your full name?

First name (1)
Last name (2)

Q3 Gender

- ❍ Male (1)
- ❍ Female (2)

Q4 What is your age (in years)?

Q5 Please indicate your predominant racial ancestry. Use percentages that add up to 100%. You can use grandparents' ethnicity to calculate the predominant racial ancestry percentage. (If you are not certain about your grandparents' ethnicity, use your parents' ethnicity). For example, if your maternal grandmother is Native American and the rest of the grandparents are European, your predominant racial ancestry percentage is 75% European. You should choose Native American racial ancestry (25%) and European racial ancestry (75%). If you are not certain about your grandparents' ethnicity, use your parents' ethnicity. For example, if your mother is Native American and father is European, you have 50% Native American racial ancestry and 50% European racial ancestry. You should choose Native American (50%) and European (50%). Your percentage value should add up to 100%. Mark your predominant racial ancestry and insert the percentage value in the appropriate boxes.

- ❑ African (1) _____
- ❑ European (2) _____
- ❑ East Asian (3) _____
- ❑ South Asian (4) _____
- ❑ Middle Eastern (5) _____
- ❑ Native American (6) _____
- ❑ Polynesian (7) _____

Q6 Eye color

- ❍ Blue (1)
- ❍ Green (2)
- ❍ Gray (3)
- ❍ Hazel (4)
- ❍ Brown (5)

Q7 Do you have hearing loss?

- ❍ Yes (1)
- ❍ No (2)

Q8 Do you have a history of ear injuries such as direct trauma to the eardrum results in severe pain and bleeding?

- ❍ Yes (1)
- ❍ No (2)

Answer If Do you have a history of ear injuries? If Yes Is Selected

Q9 Which ear was injured?

- ❍ Right (1)
- ❍ Left (2)
- ❍ Both (3)

Answer Do you have a history of ear injuries? If Yes Is Selected

Q10 Did you receive successful treatment for the ear injury?

- ❍ Yes (1)
- ❍ No (2)

Q11 Do you have a history of ear infections?

- ❍ Yes (1)
- ❍ No (2)

Answer Do you have a history of ear infections? If Yes Is Selected

Q12 Did you receive successful treatment for all the ear infections?

- ❍ Yes (1)
- ❍ No (2)

Answer Do you have a history of ear infections? If Yes Is Selected

Q13 When was your last ear infection? _____ months ago

Q14 Does high blood pressure run in your family?

- ❍ Yes (1)
- ❍ No (2)

Q15 Do you have high blood pressure?

- ❍ Yes (1)
- ❍ No (2)

Q16 Does diabetes run in your family?

- ❍ Yes (1)
- ❍ No (2)

Q17 Do you have diabetes?

- ❍ Yes (1)
- ❍ No (2)

Q18 Do you have a ringing, static, roaring, hissing or other chronic forms of tinnitus (phantom sound sensation) in your ear?

- ❍ Yes (1)
- ❍ No (2)

Answer Do you have a ringing, static, roaring or a hissing sound...If Yes Is Selected

Q19 In which ear do you have ringing, static or a hissing sound in your ear?

- ❍ Right (1)
- ❍ Left (2)
- ❍ Both (3)

Answer Do you have a ringing, static, roaring or a hissing sound... If Yes Is Selected

Q20 How frequently do you experience a ringing, static or a hissing sound in your ear?

- ❍ Always (1)
- ❍ Very Frequently (2)
- ❍ Occasionally (3)
- ❍ Rarely (4)
- ❍ Very Rarely (5)

Q23 Have you ever experienced vertigo (a spinning type of dizziness)?

- ❍ Yes (1)
- ❍ No (2)

Answer Have you ever experienced severe vertigo (a spinning type... If Yes Is Selected

Q24 How often have you experienced vertigo?

- ❍ Never (1)
- ❍ Less than Once a Month (2)
- ❍ Once a Month (3)
- ❍ 2-3 Times a Month (4)
- ❍ Once a Week (5)
- ❍ 2-3 Times a Week (6)
- ❍ Daily (7)
- ❍ Other (insert your answer in the box with ____ vertigo episodes in a given time (like 3 episodes in 4 years) (8) _____

Q25 Are you currently taking any prescribed medication?

- ❍ Yes (1)
- ❍ No (2)

Answer Are you currently taking any prescribed medication? If Yes Is Selected

Q26 Please list the prescribed medicines

1 (1)
2 (2)
3 (3)
4 (4)

Q27 Are you taking any non-prescribed medication?

- Yes (1)
- No (2)

Answer Are you taking any non-prescribed medication? If Yes Is Selected

Q28 Please list the non-prescribed medicines

1 (1)
2 (2)
3 (3)
4 (4)

Q29 Are you currently taking birth control pills?

- Yes (1)
- No (2)
- Not applicable (3)

Q30 Do you talk on a cell phone regularly?

- Yes (1)
- No (2)

Answer Do you talk on a cell phone regularly? If Yes Is Selected

Q31 How many minutes per day on an average do you talk on your cell phone?

Q32 How many years have you used a cell phone?

Q33 Which ear do you prefer for cell phone conversations?

- ❍ Right (1)
- ❍ Left (2)
- ❍ Both (3)

Answer If Which ear do you prefer for cell phone conversations? If Right Is Selected

Q34 How much do you use your right ear for cell phone conversations? (the slide bar shows percentage value)If you always use the right ear for cell phone conversations, your right ear percentage should be 100 and the left ear percentage should be 0. If you do not have an ear preference for cell phone conversations, your right and left ear percentage will be 50.

_____ Right ear-preference percentage value (1)

Answer If Which ear do you prefer for cell phone conversations? If Left Is Selected

Q35 How much do you use your left ear for cell phone conversations? (the slide bar shows percentage value)If you always use the left ear for cell phone conversations, your left ear percentage should be 100 and the right ear percentage should be 0. If you do not have an ear preference for cell phone conversations, your right and left ear percentage will be 50.

_____ Left ear-preference percentage value (1)

Q36 Do you regularly work in a noisy place where you need to shout to be heard?

- ❍ Yes (1)
- ❍ No (2)

Q37 How painful do you find loud sounds? An example of a very loud sound is that of someone striking a marching snare drum close to your ear.

_____ 0 = no painful, 100 = very painful (1)

Answer If Do you regularly work in a noisy place where you need to ... If Yes Is Selected

Q38 How many hours do you work per week in the noisy place? _____ hours/week

Q39 Do you find loud sounds painful to your ears?

- ❍ Yes (1)
- ❍ No (2)

Q40 Do you ride a motorcycle?

- ❍ Yes (1)
- ❍ No (2)

Answer If Have you used firearms for target practicing/hunting? If Yes Is Selected

Q41 Do you use hearing protection when you are using firearms?

- ❍ Always (1)
- ❍ Most of the Time (2)
- ❍ Sometimes (3)
- ❍ Rarely (4)
- ❍ Never (5)

Answer Do you ride a motor-cycle? If Yes Is Selected

Q42 On average how much time do you spend riding a motorcycle?

- ❍ Less than once a week (1)
- ❍ 2 - 4 times a week (2)
- ❍ 5-7 times a week (3)
- ❍ More than once a day (4)

Q43 How often do you use firearms?

- Never (1)
- Less than Once a Month (2)
- Once a Month (3)
- 2-3 Times a Month (4)
- Once a Week (5)
- 2-3 Times a Week (6)
- Daily (7)

Answer Do you find loud sounds painful to your ears? If Yes Is Selected

Q44 Please provide some examples where you found loud sounds painful to your ears.

1 (1)
2 (2)
3 (3)

Answer Do you find loud sounds painful to your ears? If Yes Is Selected

Q45 Do you avoid loud situations?

- Yes (1)
- No (2)

Q46 Have you used firearms?

- Yes (1)
- No (2)

Q47 List the musical instruments you play

1. Primary instrument (1)
2. Secondary instrument (2)
3. Tertiary instrument (3)

Q48 How many years have you been studying music in schools? _____ years

Q49 How many years have you been playing music (throughout lifetime)? _____ years

Q50 List the music ensembles you are attending this semester and provide average time you spend in the ensembles per week.

	List your ensembles (1)	Average time you spend (hours/week) (2)
1 (1)		
2 (2)		
3 (3)		
4 (4)		
5 (5)		

Q51 On average, how many hours do you spend practicing your instruments? _____ hours/week (Please include practice sessions and lessons)

Q52 Do you use a music player (such as MP3 player, CD player, FM radio or smart phone etc)?

- Always (1)
- Most of the Time (2)
- Sometimes (3)
- Rarely (4)
- Never (5)

Answer Do you use a music player (such as MP3 player, CD player,... If Never Is Not Selected

Q53 On average, how loud do you play music with your music player (such as MP3 player, CD player, FM radio or smart phone etc.)? "Very soft" is defined as there is no

problem following normal conversation in the presence of music. "Very loud" is defined as even a person sitting next to you would need to shout to be heard by you.

_____ 0 = very soft; 100 = very loud (1)

Answer Do you use a music player (such as MP3 player, CD player,... If Never Is Not Selected

Q54 Choose the earphones you use with your music player. (You can choose more than one option)

- ❑ Table-top speaker (1)
- ❑ Headphone (2)
- ❑ Ear-bud (3)
- ❑ In-ear (4)
- ❑ Other (please specify) (5) _____

Q55 Do you use hearing protection while playing music (in practice room, ensembles, while listening to music players etc.)?

- ❍ Never (1)
- ❍ Rarely (2)
- ❍ Sometimes (3)
- ❍ Often (4)
- ❍ All of the Time (5)

Answer Do you use hearing protection while playing music? If Never Is Not Selected

Q56 What type of hearing protection do you use? (choose appropriate answer) Choose multiple answers if you are using specific types of hearing protections for different situations. You can mention those situations in the box.

- ❑ Foam plugs (1) _____
- ❑ Ear Muffs (2) _____
- ❑ Custom plugs (3) _____
- ❑ Musician's Non Custom Plugs (4) _____
- ❑ Other (5) _____

Q57 Do you smoke tobacco on a regular basis?

- ❍ Yes (1)
- ❍ No (2)

Answer Do you smoke tobacco on a regular basis? If Yes Is Selected

Q58 What types of smoking you prefer on a regular basis?(you can choose more than one options)

- ❍ Cigarettes (1)
- ❍ Cigars (2)
- ❍ Marijuana (3)
- ❍ Other (please specify) (4) _____

Answer Do you smoke tobacco on a regular basis? If Yes Is Selected

Q59 How many cigarettes you smoke per day? _____ Cigarettes/day

Answer What types of smoking you prefer on a regular basis?If Cigars Is Selected

Q60 How many cigars you smoke per day? _____ Cigars per day

Answer Do you smoke tobacco on a regular basis? If Yes Is Selected

Q61 How recently have you smoked? _____hours before testing

APPENDIX C

INSTRUMENT CODING

Instruments	**Rating**
Piccolo, Drum Set	10
Trombone , Bass Trombone , Tuba	9
Euphonium , Saxophone, (All Saxophones) , Horn (Horn in F, French Horn) , Trumpet, Flute, Drums/Percussion	8
Organ , Clarinet	7
Electric Guitar , Voice (All Voices and Vocal)	6
Orchestral Conducting , Bass Clarinet , Bassoon	5
Oboe	4
Harp, Violin, Viola	3
Cello, Bass (String Bass, Double Bass)	2
Classical Guitar , Piano	1

APPENDIX D

ENSEMBLE CODING

Ensemble	Rating
Marching Band, Pep Band	10
Steel Drum Ensemble (Steel Drum Band, Steely Pan Steel Drum Band, Steel Band), Jazz Band (Jazz Ensemble, Jazz Big Band, Jazz Vocal) , Concert Band (Wind Ensemble, University Band, U Band, Symphonic Band, Symphony Band, College Band)	9
Horn Ensemble (Horn Choir), Sax Quartet, Trumpet Ensemble, Flute Choir, Orchestra (Symphony, University Symphony, Symphony Orchestra, Fayetteville Symphony, University Orchestra Appalachian Philharmonia, Community Orchestra), Opera Orchestra, Opera (Opera Workshop), Percussion Ensemble	8
Jazz Combo (Blues Band)	7
Chorus (University Chorale, Chorale, Chorale, Choral, Chamber Singers, Choir, Women's Glee, Men's Glee, Schola Cantorum, Opera, Opera Workshop, Treble Choir, University Singers, Women's Choir, Westminster Chamber Singers, App Chorale, Concert Choir, church Praise Team)	6
String Orchestra (Univ. String Orchestra), Repertory Orchestra (Rep. Orchestra), String Ensemble, Chamber Group (Chamber Music) Accompanying	5
Guitar Ensemble (Guitar, Guitar Orchestra), Piano Improvisation	3